Investigation of *CNR1* as a tumour suppressor gene at 6q15 in prostate cancer

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STATEMENT OF ORIGINALITY

The material presented in this thesis is the result of original work carried out by the author, Eiman GH Mohammad, at the Centre for Molecular Oncology, Barts Cancer Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, United Kingdom. All external sources have been properly acknowledged

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Abstract

Molecular genetic analysis by our team and others revealed a frequent deletion of the 6q15 region in prostate cancer (PCa), suggesting the presence of one or more tumour suppressor gene(s) (TSG) within this region, whose inactivation may play a role in prostate carcinogenesis. Previously we have shown that the cannabinoid receptor 1 (*CNR1*) gene, located at 6q15, was down-regulated in PCa cell lines and one clinical PCa sample at the mRNA level compared to normal tissue, suggesting CNR1 as a potential TSG in PCa. I, therefore, aimed to investigate whether *CNR1* may act as a TSG in prostate cancer.

I sequenced the coding and 1 kbp promoter regions of this gene in five prostate cancer cell lines, 22RV1, LNCaP, DU145, PC3 and VCaP. No mutations were found, which is consistent with these cells having a potentially functional *CNR1*. A 6bp polymorphism sequence was found on the promoter region, with the shorter allele more frequently found in cancer samples. The 6 bp polymorphism causes the addition of another transcription factor binding sites for each transcription factors (TFII-1, STAT4, c-Ets-1, Elk-1), but no association of the polymorphism with prostate cancer risk was found. Further investigation will determine its impact on the CNR1 gene translation and functionality in PCa. Mutation analysis was further performed using combined fluidigm amplification and next generation sequencing on 73 PCa clinical samples. No mutations in *CNR1* coding and promoter regions were identified.

CNR1 expression at mRNA levels was investigated in prostate cancer cell lines, 22RV1, LNCaP, DU145, PC3 and VCaP cells. Using real-time PCR analysis, I found CNR1 was highly expressed at mRNA level in 22RV1, LNCaP, DU145, while lower levels of expression were detected in PC3 and VCaP cells compared to PNT1a immortalised prostate epithelial cells. The protein expression of CNR1 receptors was investigated in PCa cell lines using different commercial anti-CNR1 antibodies. The specificity of the CNR1 antibody (Ab23703) was confirmed using two approaches including Knocked-down of CNR1 protein in prostate cancer cell line 22RV1 and inhibition of CNR1 glycosylation protein in the prostate cancer cell line LNCaP. These data confirmed that ab23703 antibody proved to be specific for CNR1 receptors and

can be used for further functional studies to investigate the role of CNR1 in prostate. Using ab23703 antibody, the expression of *CNR1* at the mRNA level generally correlated with the protein level in the prostate cancer cell line.

To further investigate the role of CNR1 as a TSG in prostate cancer cells, I employed various functional assays including cell viability (MTS), migration (Transwell), scratch-wound and colony formation assays. These studies showed that CNR1 knockdown in DU145 cell lines caused a significant increase in cell viability, cell migration and invasion. Furthermore, cell cycle analysis showed that CNR1 knockdown caused a decrease in G0/1 phases and an increase in G2/M phases of DU145 cells. These results suggest that CNR1 may be involved in the suppression of prostate cancer cell growth and invasion.

Next, I investigated whether targeting CNR1 with cannabinoid agonists would have a potential for treating prostate cancer. Addition of the CNR1 agonist (HU210) to the prostate cancer cells 22RV1, LNCaP, DU145, and PC3 resulted in a significant decrease in cell viability in a dose-dependent manner as determined by MTS assays. The reduction in cell viability induced by the cannabinoid HU210 was significant (**$P < 0.01$) and was prevented by the CNR1 antagonist (R)-SLV-319 in all evaluated cell lines, demonstrating that HU210-induced cell killing is initiated through activation of the cannabinoid receptor and that CNR1 mediates anti-proliferative effects in response to agonists. I further demonstrated that HU210 reduces the viability of the (22RV1, LNCaP, DU145, PC3) cells through induction of apoptosis by Western blot analysis of caspase-3 and PARP proteins.

My findings demonstrate that *CNR1* may play an important role in proliferation, migration and invasion of prostate cancer cells. These results suggest that *CNR1* may act as a tumour suppressor gene in prostate cancer cells.

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Abbreviations

°C: Celsius
ATCC: American type culture collection
ATP: adenosine 5'-triphosphate
AR: Androgen Receptor
Bax: Bcl-2-associated X protein
Bcl-2: B cell lymphoma 2
BSA: bovine serum albumin
cDNA: Complementary deoxyribonucleic acid
CML: Chronic myeloid leukaemia
CNR1: Cannabinoid receptor 1
Ca^{2+}: calcium
$CaCl_2$: calcium chloride
cDNA: complementary DNA
CO_2: carbon dioxide
CsCl: caesium chloride
CTL: cytotoxic T lymphocytes
CTLA4: cytotoxic T-lymphocyte antigen 4
D: day
DEPC: Diethylpyrocarbonate
DIG: Digoxigenin
DRE: Digital rectal examination
DAPI: 4', 6-diamidino-2-phenylindole
DBP: DNA binding protein
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dNTP: deoxynucleotide triphosphate
dsDNA: double-stranded DNA
EC50: Half maximal effective concentration(s)
EDTA: ethylene diaminetetraacetic acid
ER: endoplasmic reticulum
FACS: fluorescence-activated cell sorting
FADD: Fas-associated protein with death domain
FBS: foetal bovine serum
FCS: Foetal calf serum
FISH: Fluorescence in situ hybridization
FITC: Fluorescein isothiocyanate
FSC-A: forward-scattered light area
g: gram(s)
GABP: guanine-adenine-binding protein
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GFP: green fluorescent protein

GWAS: Genome-wide association studies
h: Hour
HG-PIN: High-grade prostatic intraepithelial neoplasia
HU210: [(-)3-(1,1-dimethyheptyl)-(-)11-hydroxy-Δ^8-tetrahydrocannabinoil]
H&E: haematoxylin and eosin
HCl: hydrochloric acid
HEK: human embryonic kidney
HPV: human papilloma viruses
HRP: Horseradish peroxidase
IAP: inhibitor of apoptosis
Ig: immunoglobulin
IHC: immunohistochemistry staining
IL: interleukin
Kb: kilobase
Kbp: kilobase pair (s)
KCl: potassium chloride
kDa: kilodalton(s)
l: Litre(s)
LN: Lymph nodes
Min: Minute
M: metre(s)
M: molar
mRNA messenger RNA
mTOR: mammalian target of rapamycin
MTX: mitoxantrone
MV: Measles virus
n: number of specimens per group
NaCl: sodium chloride
Neg: negative
NF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells
NK: natural killer
NP: nucleocapsid protein
OD: optical density
P: phosphoprotein
p: p-value
PCa: Prostate cancer
PSA: Prostate-specific antigen
PAGE: polyacrylamide gel electrophoresis
PARP: Poly (ADP-ribose) polymerase
PBS: phosphate-buffered saline
PCNA: proliferating cell nuclear antigen
PCR: polymerase chain reaction
pH: potentiometric hydrogen ion concentration
PI: propidium iodide
PI3K: phosphatidylinositol-3-kinase
PMS: phenazine methosulfate
Pos: positive
ppc: particles per cell

pRB: Retinoblastoma Family proteins
PS: penicillin-streptomycin
PS: phosphatidyl serine
PVDF: polyvinylidene difluoride
qPCR: quantitative real-time PCR
RFP: red fluorescence protein
RNA: ribonucleic acid
rpm: revolution(s) per minute
RPMI: Roswell park memorial institute
RT: room temperature
s: Second
S.D.: standard deviation
SDS: sodium dodecyl sulfate
SEM: standard errors of the mean
ssRNA: single stranded RNA
TAA: tumour-associated antigens
TBE: tris-borate-EDTA
TBST: tris-buffered saline with Tween 20
TCR: T cell receptor
$T_{EM:}$ effector memory
TEMED: Tetramethylethylenediamine
Trypsin-EDTA Trypsin-Ethylenediaminetetraacetic Acid
TSG: Tumour suppressor gene
UPR: unfolded protein response
UV: ultraviolet
w/v: weight/volume
WB: Western blotting

1 INTRODUCTION AND AIMS

1.1 HUMAN PROSTATE CANCER

In the adult, the prostate is a small acorn-shaped gland, with ductal –acinar histology, that lack discernible lobular organization. The prostate gland surrounds the urethra at the base of the bladder and functions by contributing secretory proteins to the seminal fluid. McNeal et al. defined three morphological regions within the human prostate: the peripheral zone, the transition zone and the central zone (Figure 1.1) (McNeal 1969).

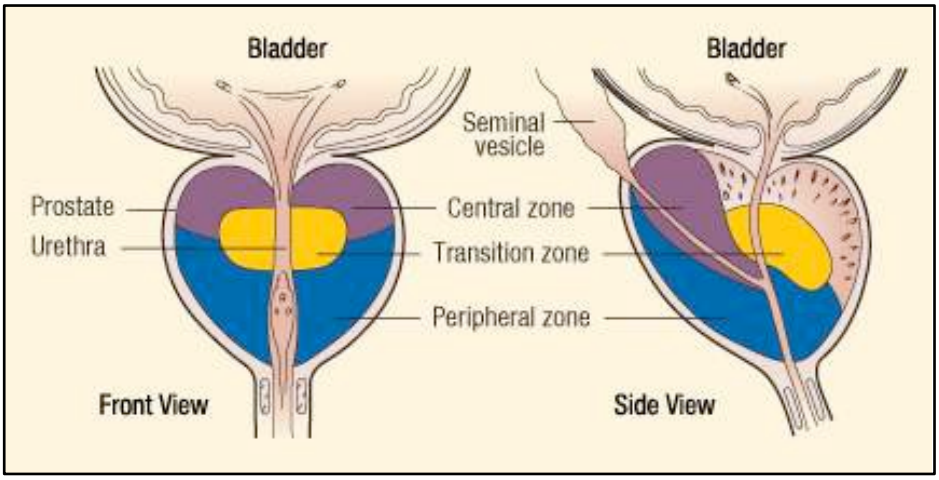


Figure 1.1 Diagram of the male prostate gland and the three distinct areas of the prostate. The peripheral zone, the central zone, and the transition zone. Adapted from http://radiologyreview.tumblr.com

Prostatic adenocarcinomas arise from the lining of the gland, more often in the peripheral zone of the prostate. The normal prostatic epithelium is arranged in a double layer basal and luminal cells along with some rare embedded neuroendocrine cells (Figure 1.2). Matrix cells and stromal fibromuscular cells transmit signals to adjust the epithelium. The epithelium is separated from the stroma by membrane containing laminins (LM5, LM10) and collagens (COLIV, COLVII). Luminal cells express high level of androgen receptor, PSA and Keratins (K8 & K18) with no integrins expression. Basal cells express high level of p63, Bcl2, EGFR, Met and

keratins (K5 & K14) and integrins (α6β4, α3β1)(de Muga, Hernandez et al. 2010). In addition, intermediate basal and luminal cells contains K5 and K18 (van Leenders, Aalders et al. 2001). The tumours of prostate are characterized by progressive loss of the basal cells and loss of the markers LM5 and COLIV. (Figure 1.2) (Frank and Miranti 2013). In addition, the tumours also express integrins, α6β1 that promotes an abnormal growth and survival of prostate cancer. Moreover, tumour cells frequently co-express basal and laminal keratins, K5 and K8 respectively. .

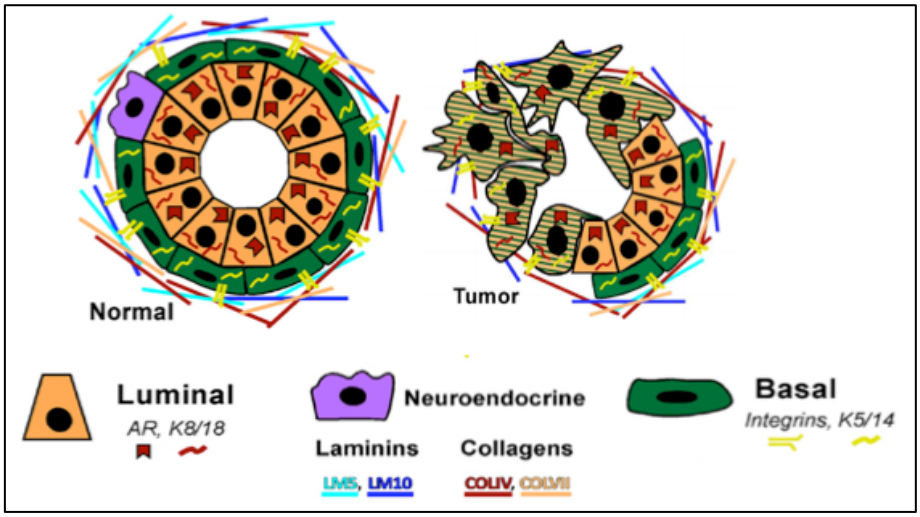


Figure 1.2 Prostate epithelial gland structure. Normal prostate epithelium is composed of a double layer of basal cells, and luminal and a few rare neuroendocrine cells. The epithelium is separated from the underlying stroma by a basement membrane. Basal cells that interact specifically with the basement membrane. Basal cells do not express AR. Adapted and modified from (Frank and Miranti 2013).

Histopathological studies of tissue from prostate cancer led to the identification of a specific type of lesion that is thought to be the main precursor of human prostate cancer, known as prostatic intraepithelial neoplasia (PIN). PIN and most advanced high grade PIN (HGPIN) is found together with cancer. Early PIN lesions are not considered as a prerequisite for cancer. However, HGPIN is considered the precursor lesion of most intermediate and high grade adenocarcinomas that arise in the peripheral zone (DeMarzo, Nelson et al. 2003). More than 95 % of

prostate cancers are adenocarcinomas (cancer of epithelia originating in glandular tissue) arising from the epithelial cells of the prostate. Of these, 70 % occur in the peripheral zone, 15-20 % in the centre, and 10-15 % in the transition zone. The cellular origins of prostate cancer are still controversial. It was reported that prostate cancer can arise from luminal cells (Goldstein, Huang et al. 2010) and based on the AR pathway activity, prostate cancer cells are more resemble the luminal cells than basel cells. However, the authors also reported that naïve basal cells could initiate acinar-type adenocarcinoma in response to oncogenic stimulation. In addition, other studies supported these findings including that basal cells from the BPH-1 prostate cell line can initiate prostate cancer after treating cells with estrogen and testosterone (Taylor, Toivanen et al. 2012). Other studies showed that using both mouse and human prostate tissue confirmed that transformed basal cells can develop malignant luminal progeny *in vivo* (Stoyanova, Cooper et al. 2013). These data suggest there are subfractions of basal cells within the tumour to ensure continuous production of malignant luminal-like cancer cells. Most cancer cells are multifocal and simultaneously developed influenced in many regions of the prostate gland, which indicates that prostate cancer is probably the result of non-clonal expansion.

1.2 PROSTATE CANCER EPIDEMIOLOGY AND RISK FACTORS

Prostate cancer is the most common non-cutaneous malignancy in western men. There were 41,736 new cases of prostate cancer in the UK in 2011, making it the fourth most common cancer in the UK. The incidence of prostate cancer is strongly correlated with age; in the United Kingdom between 2010 and 2011, approximately 85 % of cases are diagnosed in men over 65, while only 1% of the cases were diagnosed in the group of less than 50 years (www.cancerresearchuk.org). The incidence of prostate cancer has increased dramatically, likely due to increased detection through the widespread use of prostate specific antigen (PSA) and longer life (UK).

Prostate cancer is recognized as one of the major medical problems facing the male population. In UK, 10,800 prostate cancer related deaths were recorded in 2012, almost three quarter of prostate cancer deaths occur in men aged 75 and over (UK). Mortality has declined by nearly 10% in UK according to Cancer research UK (http://info.cancerresearchuk.org) and could be due to improved treatment, including new drugs such as Abiraterone, enzalutamide and more effective radiotherapy, earlier diagnosis may also contribute (Simon M Collin and Rollo Moore 2008).

Although the specific causes of initiation and progression of prostate cancer is not yet known, strong evidence suggests that both genetics and the environment play roles in the evolution of this disease. Several genetic studies suggested that hereditary factors might account for 5 - 10% of prostate cancers. The risk is increased in relatives of affected males. The risk of a man is twice as high if first-degree relatives, as a father or brother has prostate cancer, and the risk is greater (folds 5-11) if 2 or 3 first-degree relatives with prostate cancer (Turati, Negri et al. 2014). Therefore, men with a familiar prostate cancer should be screened earlier for this disease. Another risk factor for prostate cancer is increasing age. Men aged 75-79 years have about 130 times the risk of men of 45-49 years. The older the man, the greater the risk and this link with the post mortem results in about 80 % of men 80 years of age to have cancerous cells in their prostate.

Ethnicity is another significant risk factor. In UK, black Caribbean and black African men have approximately two to three times the risk of being diagnosed or dying from prostate cancer than white men, while Asian men generally have a lower risk than the national average (Kheirandish and Chinegwundoh 2011) (UK). Studies have shown that migrants from low risk to high-risk countries are experiencing an increase in the incidence, which suggests that environmental factors and lifestyle are important in the etiology of prostate cancer (Sutcliffe and Colditz 2013).

Diet has been associated with prostate cancer. An increased intake of animal fat and possibly red meat has been associated with an increased risk of prostate cancer (Joshi, Corral et al. 2012). In contrast, vegetables, fruit, antioxidants, and plant oestrogens found in soybeans reduce the risk of prostate cancer by 30-60% (Hori, Butler et al. 2011, Key 2011, Micheal F Leitzmann 2012). High intake of cruciferous vegetables containing the chemoprotective isothiocyanate sulforaphane was correlated with a diminished risk of prostate cancer (Aragon-Ching 2011, Ben Liu 2011, Hori, Butler et al. 2011).

Another potential risk factor is the hormone levels, in particular testosterone levels. A meta-analysis of previously published studies on hormonal predictors of prostate cancer concluded that men whose total testosterone level is in the highest quartile are 2.34 times more likely to develop prostate cancer (Shaneyfelt, Husein et al. 2000). However recent studies reported that prostate cancer is not related to exogenous and endogenous testosterone levels (Klap, Schmid et al. 2015, Boyle, Koechlin et al. 2016)

1.3 PROSTATE CANCER DIAGNOSIS AND STAGING

1.3.1 DIAGNOSIS

Patients with prostate cancer show few early symptoms. Severe symptoms such as haematuria, urinary obstructive and bone pain are only present in advanced disease or cancer with metastases. Blood levels of PSA, and digital rectal examination (DRE), magnetic resonance imaging (MRI) and computed tomography (CT) scan are the most common combinations of diagnostic tools for prostate cancer detection (Heidenreich, Aus et al. 2009). PSA values are not only influenced by the presence of prostate cancer, but also by various factors such as age, race and inflammation (FitzGerald, Kumar et al. 2013). Therefore, upon detection of elevated PSA levels, a biopsy is recommended to confirm the presence of this malignancy. In the UK, patients with a family history of prostate cancer are advised to have a PSA test (UK).

Although PSA screening has become widely used, there is increasing controversy regarding the limitations of its sensitivity and specificity. The association of PSA with age and other prostatic conditions, including benign prostatic hyperplasia (BPH), leads to a significant number of false-positive cases. Therefore, a more specific test to discriminate the false positive in these patients has been developed, the prostate cancer gene 3 (*PCA3)* molecular marker. *PCA3*-gene generates prostate-specific noncoding mRNA that is found overexpressed in 95% of prostate cancers (Groskopf, Aubin et al. 2006). PCA3 does not increase as a consequence of benign enlargement of the prostate or prostatitis (Groskopf, Aubin et al. 2006). Gene expression profiling studies have confirmed that PCA3 is one of the most sensitive and specific prostate cancer biomarkers (Tomlins 2014). Recently, a test detecting both PCA3 and the *TMPRSS2:ERG* fusion transcript in urine showed to have a higher sensitivity and result in fewer false-positives in prostate cancer diagnosis (Gregoire Robert 2012, Salami, Schmidt et al. 2013, Yang, Yu et al. 2016).

A more sensitive technique, transrectal ultrasound (TRUS) biopsy is recommended when DRE or PSA tests detect an abnormality (Lee and Chia 2015, Teoh, Yuen et al. 2015). Although prostate cancer can spread to a wide variety of organs, it tends to metastasise to the bones. Thus, when prostate cancer is diagnosed, a bone scan is performed using CT or MRI (Tombal and Lecouvet 2012).

1.3.2 PATHOLOGY GRADING SYSTEM

Adenocarcinoma of the prostate accounts for most prostate cancers. For the grading of prostate adenocarcinomas a few systems are available. The most widely used and generally accepted system is that proposed by Gleason (Mellinger, Gleason et al. 1967, Gleason and Mellinger 1974,

Gleason and Mellinger 2002). The Gleason score distinguishes five different grades or patterns on a scale of 1 to 5, from well differentiated to poorly differentiated (Figure 1.3) (Gleason and Mellinger 1974, Gleason and Mellinger 2002). Because prostate cancer is usually very heterogeneous, with two or more grades in a given cancer, Gleason incorporates both a primary and a secondary grade into his system. The final score is the sum of the primary and the secondary grade, ranging from 2 (1+1) to 10 (5+5) (Green, Hanlon et al. 1998, Humphrey 2004).

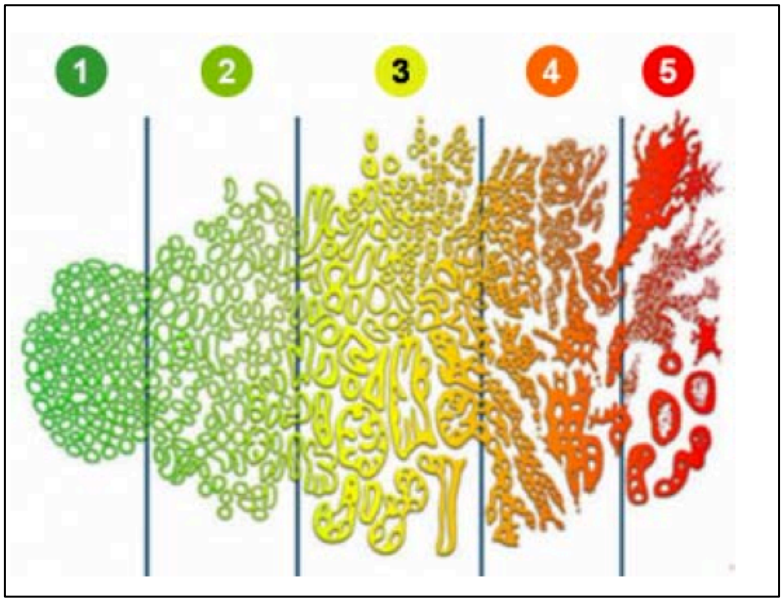


FIGURE 1.3 Gleason grade of prostate cancer. The Gleason score in five different sections: Grade 1 (well differentiated), uniform shaped glands, Grade 2 (well differentiated), some infiltration into the surrounding stroma, Grade 3 (moderately differentiated), most common grade with more variation in size, shape, and separation of the glands, Grade 4 (poorly differentiated), fusion of the glands forming a solid anastomosing network and Grade 5 (undifferentiated), characterised by a complete absence of gland formation with sheets or clusters of cells. Adapted from Dr Gleason's simplified drawing of the five Gleason grades of prostate cancer (Gleason and Mellinge.Gt 1974)

1.3.3 TMA SYSTEM FOR TUMOUR STAGE

The main goals in staging prostate cancer are to predict prognosis and to rationally select the therapy. Staging systems of prostatic carcinoma have been discussed intensively during the last few decades. In 1992 staging

concepts began to include the TNM scoring system. T (tumour) - measuring tumour size, N (nodes) - is the spread of cancer in the lymph nodes and M (metastasis) - explains spread of cancer to different distant tissues into the bloodstream. Furthermore, APC stages I-IV describes the aggressiveness of the disease, R cancer confined to the prostate designation and IV indicates a very aggressive and deadly form PC. (Table 1.1) (Liang Cheng 2012).

Table 1.1 The UICC 2002 TNM (Tumour Node Metastasis) classification is used for staging with several changes.

Stage	Tumour
I	T Primary tumour TX Primary tumour cannot be measured T0 No evidence of primary tumour T1 Clinically unclear tumour not evident by imaging
I/IIa	T1a Tumour incidental histological finding in 5% or less of resected tissue T1b Tumour incidental histological finding in more than 5% of resected tissue T1c Tumour identified by needle biopsy (e.g elevated prostate-specific antigen level)
III	T3 Tumour extends through the prostatic capsule T3a Extracapsular extension (unilateral or bilateral) T3b Tumour invades seminal vesicle(s)
IV	T4 Tumour is fixed or invades adjacent structures other than seminal vesicles: bladder neck, rectum and/or pelvic wall
	N Regional lymph nodes 3 NX Regional lymph nodes cannot be assessed N0 No regional lymph node metastasis N1 Regional lymph node metastasis
	M Distant metastasis 4 MX Distant metastasis cannot be assessed M0 No distant metastasis M1 Distant metastasis M1a Non-regional lymph node(s) M1b Bone(s) M1c Other site(s)

1.4 CLINICAL MANAGEMENT

In general, for clinically localized prostate cancer in men in good condition, with a life expectancy of 10 years or more, the goal of treatment should be

the eradication of the disease. This can be achieved by radical surgery or radiation therapy (external or interstitial) (Wilt and Thompson 2006) (Silva 2010). Active surveillance, or watchful waiting, is applied to men with a short-life expectancy or in those with a PSA test ≤ 10ng/ml, a Gleason score ≤ 6 and staged at T1c to T2a who decide not to undergo immediate surgery or radiation therapy (Damber and Aus 2008, Albertsen 2011). Tissue specimens from prostate biopsies are histologically analysed and provide information of the presence of prostate cancer cells and tumour aggressiveness (William K. Oh 2007.). Some of the tests have been commercially developed for prostate cancer but not approved by the USA FDA including Oncotype DX assay which is a multi-gene expression assay developed for FFPF diagnostic prostate needle biopsies as little as 1mm of prostate tumour. This assay measures expression of 12 cancer-related genes which are involved in four different biological pathways (*AZGP1*, *KLK2*, *SRD5A2*, *RAM13C*, *TPX2*, *FLNC*, *GSN*, *TPM2*, *GSTM2*, *BGN*, *COL1A1*, *SFRP4*) (Boström, Bjartell et al. 2015). This assay has been analytically and clinically validated as a predictor of aggressive prostate cancer. Another active surveillance test called the Decipher test (GenomeDx Bioscience), measure the expression level of 22 RNA biomarkers involved in multiple pathways that have been associated with aggressive prostate cancer (Ross, Feng et al. 2014). It predicts the probability of metastasis after prostatectomy and provides an assessment of tumour aggressiveness.

The best choice for locally advanced prostate cancer is the combination of radiation and hormonal therapy (Lars Budaus 2012). The main treatment for patients with lymph node or distant metastases is androgen-deprivation therapy (Kirby 2008, Minelli, Bellezza et al. 2009, Gupta-Elera, Garrett et al. 2012, Theodorescu 2010). However, most cases will experience the development of androgen-insensitive prostate cancer clonal expansion, also known as castration-resistant prostate cancer (CRPC). Unfortunately, there are no established treatment options for this type of prostate cancer, and palliative care is the most common choice for its management (Amaral, Macedo et al. 2012). So much remains to be done to develop treatment options for patients, since treatment of CRPC remains a major challenge.

Hormonal treatment is initially effective in most patients, using luteinizing hormone releasing hormone (LHRH) agonist leuprolide acetate (Prostap) or androgen-receptor antagonists as abarelix (Gomella 2009). But this effect is temporary, because virtually all patients inevitably develop disease progression (Mei Ka Fong 2012). Once the disease is progressive under hormonal therapy, so called hormone resistant prostate cancer, there is no curative second line of treatment available (Mei Ka Fong 2012). Patients with hormone refractory prostate cancer HRPC have limited treatment options beyond the addition of other antiandrogens.

The current treatment of choice for castration-sensitive prostate cancer (CSPC) is androgen ablation therapy or androgen deprivation therapy. This treatment is intended to block the production of androgens, which, in turn, regresses the growth, and spread of prostate cancer. Some drugs approved by the FDA include Abiraterone, a CYP17 inhibitor that leads to the reduction of testosterone levels (Pezaro, Mukherji et al. 2012), and Enzalutamide, AR antagonist (Scher , Fizazi et al. 2012). Initially, patients respond well to these treatments, but they do not achieve a complete cure, resulting in recurrence of prostate cancer and eventually death (Amaral, Macedo et al. 2012). The main challenge of hormone ablation therapy is that it does not target prostate cancer cells able to survive and proliferate in the absence of androgens (Katzenwadel and Wolf 2015).

Despite using hormone therapy, men with metastatic PCa eventually develop castration-resistant prostate cancer (CRPC). This means that the cancer is able to grow and continue to spread. In these cases, other additional treatment is required to help in controlling the growth of the cancer (Hotte and Saad 2010).

First-line chemotherapeutic treatment, Docetaxel and prednisolone are presently considered the standard of care for men with CRPC and radiologically proven metastatic disease (Petrylak , Tangen et al. 2004, Tannock , de Wit et al. 2004). This combination was recommended following the publication of two large randomised controlled trials that

compared it with the previously used standard of mitoxantrone and prednisolone. The survival rates have been shown to be significantly better with docetaxel and prednisolone, compared with mitoxantrone and prednisolone (Petrylak , Tangen et al. 2004, Tannock , de Wit et al. 2004). In addition, patients treated with docetaxel showed a greater reduction in pain (35%) compared to men treated with mitoxantrone (22%) (Petrylak , Tangen et al. 2004, Tannock , de Wit et al. 2004).

Second-line chemotherapeutic treatment, two trials, SPARC (Sternberg, Petrylak et al. 2009) and TROPIC (de Bono, Oudard et al. 2010) have assessed chemotherapeutic agents in patients with progressive disease after docetaxel. The results showed a significant and clinically relevant survival advantage with cabazitaxel therapy (de Bono, Oudard et al. 2010, Chopra and Rashid 2015, Perletti, Monti et al. 2015). Considering all these positive results, cabazitaxel was approved as a second-line chemotherapy drug in patients with CRPC and is available on the Pharmaceutical Benefits Scheme for Australian men (Chopra and Rashid 2015). Subsequently, additional trials were successful in prolonging survival in the post-docetaxel setting, including COU-301 and AFFIRM. The combination of abiraterone and prednisone (compared with placebo and prednisone) was shown to significantly prolong median overall survival. In view of these results, the US Food and Drug Administration (FDA) approved abiraterone. The COU-AA-301 trial of abiraterone in patients with mCRPC previously treated with docetaxel chemotherapy demonstrated improved overall survival (de Bono, Logothetis et al. 2011). These trials have evaluated whether, compared with prednisolone and placebo, prednisone and abiraterone acetate can improve survival. Abiraterone acetate is a potent and irreversible inhibitor of CYP17, which is a critical enzyme in androgen biosynthesis (Hotte and Saad 2010, Pezaro, Mukherji et al. 2012, Omlin, Pezaro et al. 2014). Enzalutamide is an androgen receptor signalling inhibitor (ARSI) that binds the androgen receptor ligand site and thereby inhibits nuclear translocation of the androgen receptor. The results of the AFFIRM study, comparing enzalutamide with placebo in patients previously treated with docetaxel, were recently published and showed a significant advantage in overall

survival in patients with mCRPC (Scher, Fizazi et al. 2012, Beer, Armstrong et al. 2014).

Furthermore, several treatments are approved for mCRPC patients including sipuleucel-T, an FDA-approved immunotherapy. Recent additional trials using sipuleucel-T (an autologous cellular immunotherapy targeted to prostatic acid phosphatase) and randium-223 (a bone-seeking alpha particle emitter), demonstrated an improvement of survival in patients with asymptomatic mCRPC and symptomatic mCRPC patients with bone metastasis respectively (Kantoff, Higano et al. 2010, Parker, Nilsson et al. 2013, Graff and Chamberlain 2015, Nilsson 2016).

The improvements of survival in patients with metastatic castration-resistant prostate cancer have succeeded with the use of next-generation hormonal therapy, cytotoxic chemotherapy, immunotherapy and radiotherapy. A recent study (CHAARTED Trial) showed that the combination of androgen-depravation therapy (ADT) and docetaxel resulted in better cancer control than with ADT alone (Sweeney, Chen et al. 2015). In addition, it showed a significantly longer overall survival (44.0 months) than that with ADT alone followed by docetaxel (57.6 months) in men with Castration-sensitive metastatic prostate cancer and a substantial reduction of the PSA level (Sweeny et al 2015). These findings were confirmed by another study (STAMPEDE Trial) presented by The American society of clinical oncology in 2015 (James, Spears et al. 2015) (James et al 2015).

Currently, there is lack of proof for a specific sequence of therapy. All survival-prolonging treatments are effective options, taking into account the patient status and the availability of the drug. Further research is needed in managing patients and in determining the optimal sequencing of treatments over the disease course.

1.5 GENETIC ALTERATIONS IN PROSTATE CANCER

1.5.1 GENOMIC ALTERATIONS IN PROSTATE CANCER

Prostate cancer, like other types of cancer, is a result of the accumulation of both genetic and epigenetic alterations that can lead to loss of function of tumour suppressor genes (TSGs) or overexpression of oncogenes that transform normal glandular epithelium to pre-neoplastic lesions and then invasive carcinoma (Figure 1.4) (Vinall, Chen et al. 2012). With the development of new genetic and genomic technologies, extensive studies have concentrated on the identification of genetic alterations occurring in prostate cancer (Spurgers, Chari et al. 2006, Barbieri, Bangma et al. 2013).

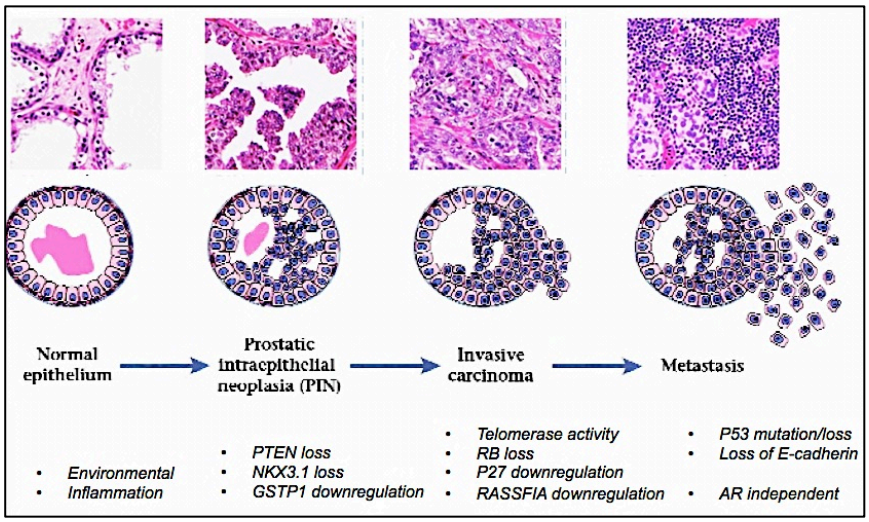


Figure 1.4 **Schematic diagram of molecular progression in prostate cancer**. Stages of progression are correlated with loss of specific chromosome regions. Adapted and modified from (Vinall, Chen et al. 2012)

Genetic alterations can be germline alterations or somatic alterations. Also, epigenetic mechanisms, including DNA methylation, contribute to prostate cancer.

1.5.2 GERMLINE MUTATIONS AND POLYMORPHISMS IN PROSTATE CANCER

As age, race, and family history remain primary risk factors for the development of prostate cancer. It has been shown that prostate cancer is one of the most heritable cancers with epidemiological studies suggesting the role of genetics in prostate cancer development (Van den Broeck, Joniau et al. 2014)

Although an increasing number of candidate genes for hereditary prostate cancer have been identified, from 5% to 10% of prostate cancer cases believed to be primarily caused by high-risk inherited genetic factors or prostate cancer susceptibility genes (Carter, Beaty et al. 1992, Lynch, Kosoko-Lasaki et al. 2016).

Prostate cancer linkage studies have been used to localise rare and highly susceptibility genes. Genes including the RNase L (*RNASEL*) gene in hereditary prostate cancer 1 (*HPC1)* on chromosome *1q23-25* (Wei, Xu et al. 2012), the elaC homolog 2 (*ELAC2*) gene in hereditary prostate cancer 2 (*HPC2*) on chromosome *17p11* (Xu, Tong et al. 2010), and the macrophage scavenger receptor 1 (*MSR1*) gene at 8p22 have been identified as prostate cancer susceptibility genes by linkage and mutational analyses (Maier, Vesovic et al. 2006). However, most gene linkages were not replicated across studies. The most promising candidate identified so far is *BRCA2*, which is associated with a 20-fold increased risk for breast cancer relative to the general population, and the evidence points to a more important role of this gene in prostate cancer at a younger age. However, this genetic linkage may explain only a small fraction of familial prostate cancer because germline mutations of *BRCA2* are quite rare in prostate cancer patients (Akamatsu, Takata et al. 2012).

The most common germline alterations described in prostate cancer are frame-shift mutation of the ribonuclease L-2, 5-oligoisoadenylate synthase-dependent gene (*RNASEL/HPC1)* located at 1q25. Two mutations in *RNASEL* (Met1Ile and Glu265X) were reported to segregate

with prostate cancer. One of these, Glu265X, was also associated with prostate cancer risk in familial and sporadic prostate cancer in other studies (Wei, Xu et al. 2012). A recent study showed the association between *RNASEL* Asp541Glu and Arg462Gln polymorphisms and prostate cancer risk (Bingbing Wei 2012).

Furthermore, a study used linkage analysis in combination with targeted massively parallel sequencing to identify a recurrent mutation in *HOXB13* that is associated with early-onset and hereditary prostate cancer (Ewing, Ray et al. 2012). This novel rare mutation (G135E) of the *HOXB13* gene was also found to be associated with increased prostate cancer risk in Chinese men (Lin, Qu et al. 2012). Recent study showed that Danish men who carry the *HOXB13* G84E mutation are more likely to develop aggressive prostate cancer (Storebjerg, Høyer et al. 2016). That finding provides further evidence that *HOXB13* plays an important role in prostate cancer etiology.

The testes, the adrenal glands and the prostate itself secrete androgens. The main androgen hormone is testosterone, which can be converted by 5-**α**-reductase to dihydrotestosterone (DHT) in prostate cells. Androgen production is regulated by endocrine stimulation from the hypothalamus via the pituitary gland. Gonadotrophin releasing hormone (GnRH) is normally released in pulses from the hypothalamus, stimulating pulsatile release of follicle-stimulating hormone (FSH) and luteinising hormone (LH), the latter stimulating production of testosterone by the Leydig cells in the testes (Figure 1.5).

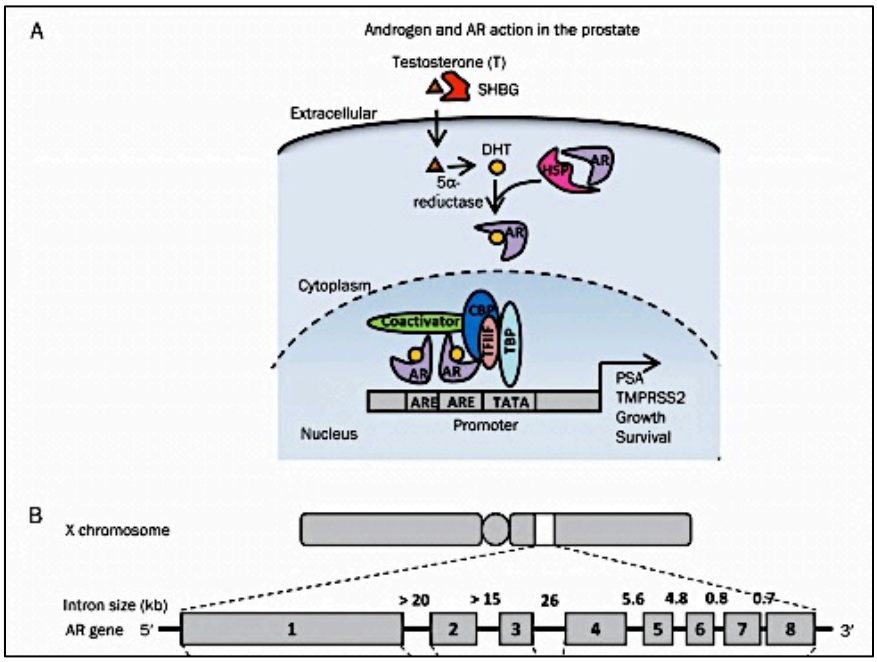


Figure 1.5 Androgen and AR action. The genome organization of the human androgen receptor gene and the functional domain structure of the androgen receptor protein. (A) Androgen and AR signaling in prostate cells. Testosterone is transported to the prostate and converted to dihydrotestosterone (DHT) by 5-α-reductase. DHT binds to the ligand-binding pocket and promotes the separation of heat-shock proteins (HSPs) from the AR. The AR then transfer to the nucleus, binds to the androgen response element (ARE) in the promoter region of target genes such as prostate-specific antigen (PSA) and TMPRSS2. The AR binds the basal transcription machinery proteins including TATA-box-binding protein (TBP), transcription factor IIF (TFIIF), other coactivators and cAMP-response element-binding protein (CREB)-binding protein (CBP), at the promoter region, SHBG: serum sex hormone-binding globulin. (B) The androgen receptor gene located on the long arm of the X-chromosome (locus: Xq11-q12). Adapted from (Tan, Li et al. 2015)

The androgen receptor (*AR*) gene, a member of the steroid and thyroid hormone receptor gene superfamily, is a transcription factor that mediates testosterone and dihydrotestosterone (DHT) activities by initiation of transcription of androgen-responsive genes. The AR gene is located on the X chromosome at the locus Xq11-Xq12. It contains eight exons interrupted by introns of varying lengths (0.7–2.6 kb) with two highly polymorphic trinucleotide repeat segments, $(CAG)_n$ and $(GGN)_n$, that reside in the transactivation domain of *AR* (Tan, Li et al. 2015).

Several studies have reported an association between prostate cancer susceptibility of the polymorphic CAG repeats in the AR gene, which codes for a polyglutamine (PolyQ) tract in the N-terminal domain (exon 1) of the AR protein (Gu, Dong et al. 2012). The role of the variation in the CAG repeats (rs4045402) length in prostate cancer risk has been investigated in many studies. A meta-analysis of 19 studies including Caucasian, African-American and Asian subjects predicted an increased risk of prostate cancer in men with shorter (≤21) CAG repeats. In addition, a Swedish study suggests that men with shorter AR CAG lengths (e.g., ≤22 repeats) are at a greater risk of developing prostate cancer. Other studies found no association between the AR CAG repeat length and prostate cancer risk. Although evidence that mutations in the AR predispose men to prostate cancer is undisputed, AR NTD CAG repeats length association with prostate cancer risk thus remains controversial (Tan, Li et al. 2015). Other genes with different polymorphisms which may influence the risk of prostate cancer are *CYP11A1 (Yamada, Nakayama et al. 2012), CYP17 (Song, Tao et al. 2016)*, *TA* dinucleotide repeat in *SRD5A2 (Choi, Kim et al. 2015)*, *HSD3B1* (Wu, Huang et al. 2015), *GSTM1* (Wang, Li et al. 2015), *GSTP1 & GSTT1 (Gong, Dong et al. 2012)*, *MYC* (Li, Liu et al. 2015), vitamin D receptor (*VDR*) levels (Jingwi, Abbas et al. 2015).

There has been an increasing focus on the role of single nucleotide polymorphisms (SNPs) in the development and progression of prostate cancer but also on their role in diagnostics and risk prediction. SNPs are the most common polymorphisms in the genomes of many species. The definition of a SNP is a variation of the DNA sequence at a frequency larger than 1% of the allele of a population (Van den Broeck, Joniau et al. 2014).

Genome-wide association studies (GWAS) provide stronger power to detect small to modest effects on disease risk. In the last decade, GWAS have successfully identified roughly 100 loci associated with prostate cancer risk (Gudmundsson, Sulem et al. 2007, Yeager, Orr et al. 2007, Thomas, Jacobs et al. 2008, Eeles, Kote-Jarai et al. 2009, Takata,

Akamatsu et al. 2010, Haiman, Chen et al. 2011, Kote-Jarai, Olama et al. 2011, Schumacher, Berndt et al. 2011, Boyd, Mao et al. 2012, Xu, Mo et al. 2012, Amin Al Olama, Kote-Jarai et al. 2013, Eeles, Olama et al. 2013, Al Olama, Kote-Jarai et al. 2014) and 33% of familial risk is associated with known SNPs (Al Olama, Kote-Jarai et al. 2014).

The great majority of GWA studies of prostate cancer have been mainly performed on European population (Amundadottir, Sulem et al. 2006, Eeles, Olama et al. 2013, Yang, Yu et al. 2016). However, in the last few years, a number of meta-analysis of GWAS data and the replication studies have showed certain loci contribute to prostate cancer in multiple ethnic populations such variants at 11q12 (rs1938781), 10q26 (rs2252004) and 3p11.2 (rs2055109) in Japanese populations (Akamatsu, Takata et al. 2012), loci at 9q31.2 (rs817826) and 19q13.4 (rs103294) in Chinese populations (Xu, Mo et al. 2012), at loci at 8q24 (rs114798100 and rs111906923) in African ancestry (Han, Rand et al. 2016). Recently, Marzec et al have shown that four loci (rs12567052, rs10235505, rs7463708 and rs1456315) on 8q24 contribute to prostate cancer risk in both European and Chinese populations (Marzec, Mao et al. 2016).

Numerous studies have been reported the association of SNPs in 8q24 region and prostate cancer. To date there are about 64 variants of 8q24 SNPs investigated for the association with prostate cancer risk, and only 20 of those are confirmed to be prostate cancer risk SNPs (Amundadottir, Sulem et al. 2006, Gudmundsson, Sulem et al. 2007, Gudmundsson, Sulem et al. 2007, Haiman, Patterson et al. 2007, Witte 2007, Yeager, Orr et al. 2007, Yeager, Chatterjee et al. 2009, Li, Liu et al. 2015, Ren, Zhang et al. 2015, Bishop, Han et al. 2016, Han, Rand et al. 2016, Marzec, Mao et al. 2016). For example, it was confirmed that SNPs in three 8q24 regions (rs1447295, rs1690979 and rs6983267) were significantly associated with prostate cancer risk in Caucasian families (Salinas, Kwon et al. 2008). Most recently, Marzec et al have found that mainly four loci on 8q24 contribute to prostate cancer risk in a large population of Chinese men, which is different in the European population (Marzec, Mao et al. 2016). Other studies showed similar results among Caucasians, Asians

and African-Americans (li, Liu et al. 2015). Moreover, a meta-analysis for rs6983267 polymorphism in prostate cancer was performed, and data showed that rs6983267 is significantly associated with prostate cancer risk in both European and Asian descent (Zhu, Zhang et al. 2015).

Several other studies showed that SNPs is associated with prostate cancer aggressiveness. Williams et al, identified SNPs in three genes associated with poor clinical outcomes in TCGA (Provisional) and GSE21032 prostate cancer gene expression datasets (*CXCL14*, *ITGAX*, and *LPCAT2*) were all associated with aggressive prostate cancer including the following SNPs, rs2237061, rs10515473 and rs4463175 in *CXCL14* gene, were found to be associated with Gleason score at prostatectomy (Williams, Lee et al. 2014). In addition, Berndt et al, identified two loci specific for aggressive prostate cancer, rs78943174 at 3q26.31, and rs35148638 at 5q14.3, which are associated with Gleason score, a pathological measure of prostate cancer aggressiveness. These SNP are located near genes involved in vascular disease, cell migration and metastasis, which make them interesting loci for further study (Berndt, Wang et al. 2015). Furthermore, The NCI-SPORE Genetics Working Group provided further support using clinicopathologic information and genotype data for 36 SNPs. They showed that a prostate cancer-risk SNP rs2735839 near the *KLK3* gene on chromosome 19q13 might be associated with aggressive and high-grade prostate cancer (Helfand, Roehl et al. 2015). All in all, these findings may improve the understanding of prostate cancer susceptibility and provide clues for further functional studies.

1.5.3 SOMATIC GENOMIC ALTERATIONS IN PROSTATE CANCER

It has been clear for many years that somatic alterations of the genome occur at a high frequency in the majority of cells isolated from most common forms of cancer.

Prostate cancer cells usually contain a large number of somatic genome alterations, such as, deletions, point mutations amplifications and translocations that contribute to the cancer phenotype. For example, inactivation or loss of a tumour suppressor gene by mutation or deletion, results in the loss of the tumour suppressor function, resulting in the uncontrolled proliferation of cells (Dong 2001).

Several methods such as conventional G-banding, fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) have all been performed to study chromosomal abnormalities in prostate cancer. Various chromosomal abnormalities have been described in prostate cancer. The most commonly reported are gains of 2p, 3q, 7q, 8q, 9q, 17q, 20q, and Xq, deletions of 2q, 5q, 6q, 8p, 10q, 12p, 13q, 16q, 17p, 17q, 18q, 21q, and 22q, hyperdiploidy, and aneusomy of chromosomes 7 and 17 (Gu and Brothman 2011, Williams, Greer et al. 2014).

Recent studies using next-generation sequencing (NGS) have provided a catalogue of such changes at much higher resolution than has previously been possible. Besides small sequence changes (such as point mutations, and small insertions or deletions of nucleotides), NGS studies highlight the abundance of chromosomal aberrations in cancer genomes. These chromosomal aberrations include different forms of abnormalities, such as chromosomal rearrangements (e.g. large insertions or deletions, rearrangement between chromosomes, chromosome fragmentation) and regions of amplification, as well as numerical aberrations associated with the gain or loss of whole chromosomes (or aneuploidy)(Ashok 2014).

With the development of NGS technology, prostate cancer is being deeply exposed in terms of diagnosis and prognosis at the level of its genetic basis. New prospectus of personalised treatment is arising due to the latest developments in NGS tools, which can identify genomic mutations and their downstream effects on gene expression (Yadav, Li et al. 2015). NGS tools can reveal genetic alterations at the level of the whole genome

(Whole genome sequencing; WGS), exome (Whole-exome sequencing; WES) and transcriptome (RNA-Seq). Information can then be combined with clinicopathological and radiological data leading to an accurate diagnosis of cancer aggressiveness (Yadav, Li et al. 2015).

Genomic alterations such as insertions, deletions, rearrangement of repetitive elements, copy number variations, microbial infections, active retrotransposons, chromosomal translocations and inversions can be inclusively provided by WGS. In addition, WGS can be a sufficient advancement in providing a patient-specific reference of genomic alterations to track tumour progression, treatment efficiency and mechanisms of drug resistance (Yadav, Li et al. 2015). On the other hand, accurate detection of mutations by WGS demands great amounts of DNA, as, opposite to exome sequencing, WGS does not involve a DNA amplification step, in addition to costly expenditures. Whereas WGS is considered a useful tool in diagnostics (Majewski, Schwartzentruber et al. 2011, Rabbani, Tekin et al. 2014), since it analyses approximately 2% of genomic DNA referred to the exome with high sensitivity. However, WES is insufficient in recognizing copy number variations and other mutations that are included in the whole genome.

Several of WES-based studies have identified genes that are recurrently mutated, such as, Barbieri et al. 2012 have sequenced the exome of 112 prostate tumour and normal tissue pair and identified genes that are recurrently mutated in prostate cancer including *SPOP*, *MED12* and *FOXA1* genes, using whole-exome sequencing (Barbieri, Baca et al. 2012). In addition, the *SPOP* gene lacked *EST* family gene rearrangement, which may anchor a district subtype of *EST* fusion-negative prostate cancer (Barbieri, Baca et al. 2012, Baca, Prandi et al. 2013, Barbieri, Bangma et al. 2013). Furthermore, Grasso et al, 2012 sequenced the exome of 50 CRPC tissues and identified the recurrent alterations in genes knows to be associated with prostate cancer including *TP53*, *AR*, *ZFHX2* and *PTAN* (Grasso, Wu et al. 2012). Recently, Robinson et al have used whole-exome and transcriptome sequencing on 150 mCRPC tissue tumour biopsies. And found abnormalities in *AR*, *EST*,

TP53 and *PTEN* genes. Moreover, aberrations were found in high frequencies in *BRCA2*, *BRCA1* genes compared to the normal those in the primary prostate cancers (Robinson, Van Allen et al. 2015). Other studies identified copy number alterations involving *MYC*, *RB1*, *PTEN*, and *CHD1* (Berger, Lawrence et al. 2011, Pflueger, Terry et al. 2011, Gao, Vela et al. 2014).

The complexity of prostate cancer, both diagnostic and clinical management is based on its multifocal origins within the prostate body. Several studies have shown that independent clonal multiple tumour foci occur. These different tumour foci, in turn, can advance at different rates depending on the nature of the genetic alterations that confer different degrees of biological aggressiveness (Taylor, Schultz et al. 2010, Lindberg, Klevebring et al. 2013, Cooper, Eeles et al. 2015). A research team recently found that a prostate tissue that appears normal might not be that normal after all (Cooper, Eeles et al. 2015). They performed the whole genome DNA sequencing technology to read the complete DNA code inside the cells present in all the biopsies that were taken from the prostates of three men. They found that the so-called normal prostate cells, that surrounded the tumour cells, harboured genetic alterations in their DNA that might develop into cancer. 48 % of the so called normal prostate samples carried genetic mutations and genetic errors, strongly supporting the idea to work on the whole prostate rather than focusing on one single area.

WGS-based studies have also identified genes that involved in prostate cancer including recent studies have uncovered biallelic loss of *BRCA2* gene in sequenced CRPC samples (Cheng, Pritchard et al. 2015, Decker, Karyadi et al. 2016). This discovery is potentially important prognostic and biomarkers for treatment response to DNA-damaging therapy, chemotherapies and in aggressive prostate cancer.

NGS applications have also been used to screen prostate cancer for multiple gene alterations in men routinely diagnosed with this disease.

Beltran et al. (Beltran, Yelensky et al. 2013) performed targeted sequencing using advanced prostate tumour FFPE samples (50 ng DNA). They observed that over 44% of CRPCs harboured genomic alterations involving the *AR* gene. Other recurrent mutations included *TMPRSS2-ERG* fusion, *PTEN* loss, *TP53* mutation, *RB* loss, *MYC* gain and *PIK3CA* mutation, *BRCA2* loss and ataxia telangiectasia mutated gene (*ATM*) mutations. Recently, a study was performed using small amounts of DNA (30 ng) dissected from transrectal ultra-guided (TRUS) or transperineal needle biopsy specimens. It showed that this amount of DNA was sufficient to generate mutation data using NGS technologies. The results were consistent with other studies observed in prostate cancer (Beltran, Yelensky et al. 2013, Iacono, Buttigliero et al. 2016) including *TMPRSS2-ERG* fusion, *TP53*, *ATM*, and *SPOP* (Manson-Bahr, Ball et al. 2015). This technique provides a more robust method that may be used to assess gene mutations in men underdoing diagnosis for prostate cancer.

NGS applications are further used to pinpoint alterations at the level of gene expression and detect new splice variants and SNPs. Identification and quantification of allele-specific expression, non-human transcripts, deregulated gene fusions are also enabled by the use of RNA-Seq tools although it is a challenging process due to RNA fragility and many preparatory steps that demand matching process to the reference genome (Fang and Cui 2011, Han, Vickers et al. 2015). In addition, identification of methylated CpG islands are also of great interest to target down-regulated genes in both normal and cancerous cells by NGS using a technique known as reduced representation bisulfite sequencing (RRBS) (Smith IM et al 2010)(Delpu, Cordelier et al. 2013).

1.5.3.1 Gene fusions in Prostate cancer

Gene fusions are important in the development of many haematological malignancies and sarcomas but are rare in most other tumour types (Parker and Zhang 2013). Fusion genes may involve the regulatory elements of one gene (often tissue-specific) aberrantly apposed to a proto-oncogene, for example, immunoglobulin and T-cell receptor regulatory regions fused to the MYC oncogene in B and T cell

malignancies, respectively. Alternatively, coding regions of two genes are juxtaposed, resulting in a chimeric protein with a new or altered activity, for example, the BCR–ABL1 gene fusion in chronic myeloid leukaemia (CML) and a subset of acute lymphocytic leukaemia (Kumar-Sinha, Tomlins et al. 2008). The BCR–ABL1 gene fusion on the Philadelphia chromosome (aberrant chromosome 22), discovered by Nowell and Hungerford in 1961, results from a translocation of the proto-oncogene ABL1 from chromosome 9 to the BCR gene on chromosome 22. BCR–ABL1 is a diagnostic marker for CML. Detection of BCR–ABL1 fusion transcript in peripheral blood is used to confirm CML diagnosis, and to monitor cytogenetic remission and residual disease (Kumar-Sinha, Tomlins et al. 2008)

In 2005, Petrovics et al (Petrovics G 2005) reported frequent overexpression of the *ETS* transcription factor *ERG* mRNA in clinical prostate cancer (62% of 114 prostate cancer samples). Later that year, Tomlins et al (Tomlins SA 2005) showed that *ERG* overexpression in prostate cancer was caused by a recurrent gene fusion of the *TMPRSS2* gene to the *ERG* and *ETV1* genes. The microarray-expression profiles studies revealed strong outlier profiles for two *ETS* family transcription factors genes, *ERG* and *ETV1* (Tomlins SA 2005)

Since the discovery of *TMPRSS2:ERG* and *TMPRSS2:ETV1* fusions, subsequent studies have identified additional transcripts from the fusion of *TMPRSS2* and the *ETS* variants *ETV4*, *ETV5* and *ELK4*. These transcription factor gene family members have been identified as common events in prostate cancer and highlight the importance of fusion genes in the development and progression of epithelial cancers. *TMPRSS2:ERG* gene fusion, which accounts for approximately 85% of all the *ETS* fusion samples, has been reported in more than 50% of early- and mid-stage localised and hormone refractory metastatic prostate cancers (Mosquera, Perner et al. 2008)

Recently published data in our group showed that *TMPRSS2: ERG* occurs

less frequently in Chinese prostate cancer samples than in western prostate cancer samples. We found a significant reduction in the frequency of certain somatic genomic changes that are commonly found in western prostate cancers, including the 21q22.2-22.3 deletions, which involve the *TMPRSS2:ERG* fusion gene, and 10q deletion, which causes *PTEN* inactivation (Mao, Yu et al. 2010, Xue, Mao et al. 2012). Low *TMPRSS2:ERG* frequency has been found in other Asian populations (Chen, Ren et al. 2014, Kelly, Kong et al. 2015).

1.5.4 EPIGENETIC CHANGES IN HUMAN PROSTATE CANCER

Epigenetic is the study of heritable changes in gene expression that are not explained by changes in DNA sequences. Three important mechanisms lead to epigenetic events: DNA methylation, histone modification, and RNA-associated silencing. DNA methylation and histone modification play an essential role in many molecular and cellular alterations associated with the development and progression of prostate cancer (Kim and Yu 2012).

Aberrant epigenetic events such as DNA hypermethylation, DNA hypomethylation and histone acetylation have been observed in prostate cancer (Vasiljević, Wu et al. 2011, Kim and Yu 2012). The most common epigenetic change in prostate cancer is hypermethylation of glutathione-S-transferase proteins (*GSTPs*) occurring in approximately 70% of PIN lesions and in the majority (>90%) of prostate carcinomas but not in normal prostate tissue and benign prostatic hyperplasia. Abnormal gene methylation has been associated with poor clinical outcomes in patients with prostate cancer and it may serve as a potentially useful tool for disease diagnosis and prognosis (Carmen Jero´nimo 2011). A recent report showed that the DNA promoter hypomethylation of CD147 may be one of the regulatory mechanisms involved in the cancer-related overexpression of CD147 and may play a crucial role in the tumourigenesis of prostate cancer (Liang, Mo et al. 2015).

Another common epigenetic change in prostate cancer is *RASSF1A*, located at 3p21.3, which encodes a protein similar to the *RAS* effector proteins. In prostate cancer, *RASSF1A* gene silencing is observed in over 70% of cases (Nelson, De Marzo et al. 2009). Silencing of the *RASSF1A* promoter through methylation is associated with advanced grade prostatic tumours, suggesting a correlation between loss of *RASSF1A* expression and tumour prognosis (Karishma S Amin 2012). Moreover, large numbers of PIN samples show *RASSF1A* promoter hypermethylation (Li L 2004). Table 1.2 summarises other genes affected by epigenetic changes in prostate cancer.

Table 1.2 List of genes affected by epigenetic changes in prostate.

Epigenetic aberration	Gene	Function
DNA hypermethylation	*AR, ESR1, ESR2,*	Hormone signalling
	CCND2, CDKN2A	Cell cycle control
	APC, RASSF1, CDH1,	Tumour invasion
	CDH13, CDH1, CD44	Cell Adhesion
	GSTP1, MGMT	Repair of DNA damage
DNA hypomethylation	*CAGE, HPSE, PLAU*	
Histone methylation	*GSTP1, PSA*	

Adapted from (Albany, Alva et al. 2011).

1.5.5 TUMOUR SUPPRESSOR GENES INVOLVED IN PROSTRATE CANCER

The growth of cells has to be controlled by many external and intrinsic signals to maintain a steady state (homeostasis). Failure of growth inhibition is one of the fundamental alterations in the process of carcinogenesis. The proteins that prevent the cell proliferation are the products of tumour suppressor genes (TSG) (Vogelstein, Papadopoulos et al. 2013). TSGs refer to genes whose loss of function results in the

promotion of malignancy. They normally function as negative regulators of growth or other functions that may affect invasive and metastatic potential, such as cell adhesion and regulation of protein activity (Hanahan and Weinberg 2011, Logtenberg and Boonstra 2013).

According to Knudson's "two-hit" theory of TSG inactivation, the first hit is usually a point mutation or submicroscopic deletion in the first allele, followed by the second hit that affects the second allele (Knudson 2001). The second hit may result from different genetic mechanisms, such as the loss of the whole chromosome by mitotic non-disjunction, chromosomal translocation followed by the loss of a part of the chromosome harboring the TSG, mitotic recombination and subsequent selection, or deletion of the segment that harbors the TSG (Devilee, Cleton-Jansen et al. 2001). Consequently, in these examples, the second hit generates the loss of heterozygosity (LOH) in a chromosomal segment spanning the TSG, which can be used as a tool for mapping TSGs by using polymorphic loci in the region (Figure 1.6).

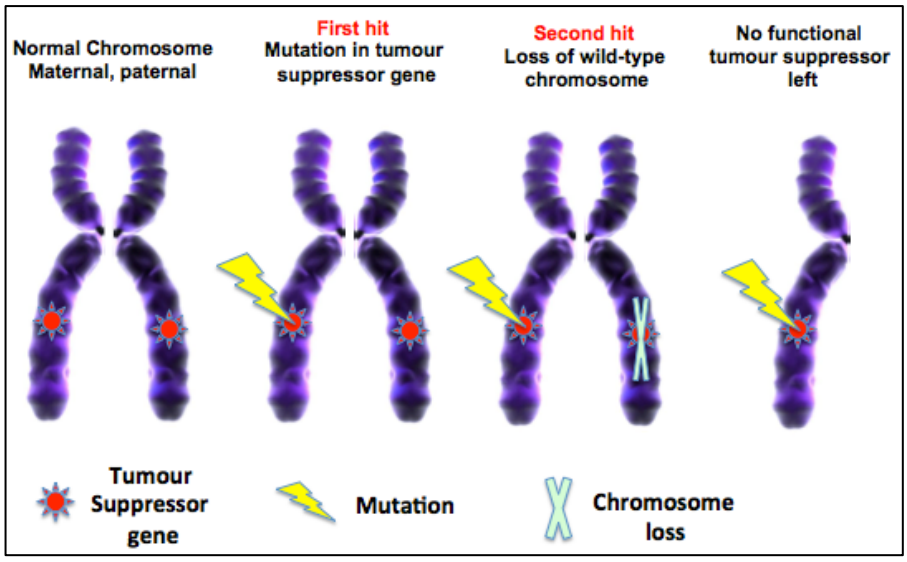


Figure 1.6 Knudson's two-hit hypothesis for tumourigenesis involving a tumour suppressor gene (TSG).

In prostate cancer, the series of genetic events underlying tumourigenesis is still poorly defined but inactivation of multiple tumour suppressor genes appears to be a common genetic alteration (Barbieri, Bangma et al. 2013).

One of the most common chromosomal deletions is the loss of 8p region, which harbours the gene *NKX3.1* (Eeles, Kote-Jarai et al. 2009, Gu and Brothman 2011). The 8p deletion was found in the HGPIN stage of prostate cancer which is considered to be a premalignant stage of prostate cancer (Dong 2006). About 40% of prostate carcinomas involved the loss of 8p according to CGH studies. At least two minimally deleted regions, 8p21 and 8p22, have been identified, suggesting that several tumour suppressor genes may be located at 8p (Abate-Shen, Shen et al. 2008).

Another frequently deleted chromosomal region in prostate cancer is the 10q23 where the well-known tumour suppressor gene *PTEN* is located. Early studies using the loss of heterozygosity analyses estimated that 35–58% of 10q deletions in advanced prostate cancers include *PTEN* (Yoshimoto, Ludkovski et al. 2012). Studies revealed that *PTEN* loss also frequently occurs in HGPIN (Yoshimoto, Cutz et al. 2006, Luchman, Benediktsson et al. 2008), suggesting that *PTEN* mutation is an early event in prostate carcinogenesis and may have an important role in cancer initiation (Mao, Yu et al. 2010, Boyd, Mao et al. 2012).

The loss of 13q has been associated with high-grade or metastatic tumours where the *RB1* gene is located. The loss of *RB1* gene on 13q14.2 is reported in approximately one-third of localized prostate cancers (Williams, Greer et al. 2014).

Deletion of 6q is not only frequently found in prostate cancer but also has been implicated in breast, ovarian, urothelial melanoma-cell leukemia, pancreatic and small cell lung cancers. Most of these studies showed multiple regions of loss at 6q (Verhagen, Hermans et al. 2002, Lane, Strefford et al. 2007, Mao, Yu et al. 2010, Shan, Ambroisine et al. 2010,

Wu, Shi et al. 2012). Deletion of 6q14–q22 is one of the most common in many cancers including prostate cancer. *SNORD50A* gene located at 6q14 was found to have a 2-bp deletion in 10% of a sporadic prostate cancer (Dong, Rodriguez et al. 2008). This gene was found transcriptionally downregulated in prostate cancer cells.

Although 6q15 deletion has been widely recognised as a frequent event in prostate cancer (Liu, Chang et al. 2007), the target TSG(s) located in this region are yet to be identified. Liu et al. (Liu, Chang et al. 2007) applied SNP array analysis on 55 matched prostate cancer and non-malignant tissue samples and identified a minimum overlapped deletion region between 90,3493 and 91,310 Kb (817 Kb), which was shared by 20 tumours. Five genes (*MAP3K*, *CASP8A2*, *CX62*, *MDN1* and *BACH2*) were located in this small region. Further analysis of *MAP3K7* revealed the correlation of its down regulation with high-grade prostate cancer (Liu, Chang et al. 2007). Carmer et al described *MAP3K7* as a prostate cancer putative TSG that encodes TGF-β activated kinase-1 (*Tak1*), which has an important role in proliferation and invasion. They showed that *Tak1* expression was progressively lost with increasing Gleason grade, both within each and across all cancers (Wu, Shi et al. 2012).

Cannabinoid receptor 1 (*CNR1)* is one of the two-cannabinoid receptors responsible for the psychoactive effect of marijuana. CNR1 is located at the 6q15-deleted region. Several reports have demonstrated that CNR1 expression was silenced in human cancer including colorectal (Bedoya, Rubio et al. 2009) and esophageal (Bedoya, Meneu et al. 2009). However, noetailed analysis of this gene had previously been reported regarding the role in prostate tumourigenesis.

Table 1.3 Deleted chromosomal regions and genes in prostate cancer

Location	Gene	Function	Status in prostate cancer	References
1p36	*EPHB2*	Tumour suppressor gene encoding a receptor tyrosine kinase	Frameshift mutations	(Huusko, Ponciano-Jackson et al. 2004)
6q14-15	*SNORD50A*	Guide RNAa in the site specific ribose methylation of preribosomal RNA	Deletion	(Dong, Rodriguez et al. 2008)
6q15	*MAP3K7/TAK1*	Putative tumour suppressor gene encodes TGF-b activated kinase-1 (*Tak1*), role in proliferation and invasion	Deletion	(Liu, Chang et al. 2007)
7q36	*EZH2*	Histone methyltransferase involved in maintaining the transcriptional -Repressive state	Deletions, missense /frameshift mutations	(Carmen Jero´nimo 2011)
8p	*NKX3.1*	Tumour suppressor regulating proliferation Of glandular epithelium	Chromosome deletion	(Bethel, Faith et al. 2006)
10p15	*KLF6*	Zing finger transcription factor associated with cell proliferation.	LOH and mutation	(Narla, Heath et al. 2001)
10q21	*ANXA7*	Ca2+-activated GTPase	Chromosomal loss	(Srivastava, Torosyan et al. 2007)
10p23	*PTEN*	Tumour suppressor by negatively regulating AKT/PKB signalling pathway	Deletion/ frameshift mutations	(Li, Yen et al. 1997)
12p12-13	*CDKN1B (p27/Kip)*	Inhibit cyclin-dependent kinases and block cell proliferation.	Deletion	(Dong 2001)

Location	Gene	Function	Status in prostate cancer	References
13q21	*KLF5*	Transcription factor associated with cell proliferation, differentiation and carcinogenesis	Hemizygous deletion	(Chen, Bhalala et al. 2003)
16q22	*ATBF1*	Control cell proliferations, up regulation of p53	Deletion/ frameshift mutations	(Sun, Frierson et al. 2005)
17p13	P53	Tumour suppressor gene involved in the induction of cell cycle arrest, apoptosis, DNA repair	Single point mutations	(Taylor, Schultz et al. 2010)
22q12	*CHEK2*	Regulate p53 in the DNA-damage-signalling pathway.	Truncation/ frameshift mutations, Deletion	(Dong, Wang et al. 2003)

1.5.5.1 Tumour suppressor gene therapy for cancer

Because tumour suppressor genes are often mutated, deleted or epigenetically silenced in cancer cells, effective anticancer therapies that target tumour suppressor genes must restore the normal functions of tumour suppressor genes. Identifications of oncogenes and tumour suppressors have played a pivotal role in enhancing our understanding of the unique biology of cancer, as well as aiding in the development of new cancer therapies. Therefore, gene therapies have been developed for cancers, where the loss of tumour suppressor functions is compensated via ectopic expression of wild-type genes. Alternatively, numerous inhibitors have been identified to either disrupt interactions between tumour suppressor genes and their negative regulators, or induce synthetic lethality, although the effectiveness of these inhibitors needs to be validated with solid clinical data. A tremendous amount of financial resources and manpower have been invested to understand the malignant nature of cancer in hopes of finding a cure.

Drug treatment for cancer depends on the notion that mutations that give rise to the development of cancer also bring about a weakness that can be exploited therapeutically (Weinstein et al. 2013). These genetic alterations consist of gain-of-function mutations in which genes are amplified, translocated, or mutated, and loss-of-function mutations in which gene function is compromised by missense mutation or deletion. The former group of mutations has been the subject of intense focus by the pharmaceutical industry for the development of targeted cancer drugs. These efforts have resulted in a number of cancer drugs that target activated driver oncogenes, such as HER2, BCR-ABL, EGFR, and BRAF (Pagliarini et al. 2015). These drugs target signaling proteins that are aberrantly activated as a direct consequence of an oncogenic mutation, and hence their inhibition is detrimental to the cancers (Weinstein 2002).

From a drug discovery perspective, the loss-of-function mutations are much harder to tackle, and the same is true for a number of activated oncogenes that have proven to be more or less undruggable, such as the

MYC transcription factor and the RAS proteins. Synthetic lethality provides the possibility of drugging undruggable targets indirectly (Prahallad & Bernards 2015).

Synthetic lethality refers to a genetic principle in which the combination of two genetic disorders is fatal, while in each individual this is not the case. Such redundancies in signaling ensure that cells often survive when a single gene is inhibited because another gene can compensate for it functionally. However, the inhibition of these compensatory genes can induce cell death, in particular, when the first gene is mutated but does not affect the growth of cells lacking that mutation. When the inhibition of a signaling pathway leads to the biochemical activation of a second pathway that mediates survival, the simultaneous inhibition of both pathways can cause cell death due to synthetic lethal interaction (Blomen et al. 2015).

There are two important aspects to synthetic lethality in the context of cancer drug development. Firstly, the genes that are synthetic lethal with oncogenic driver mutations are not necessarily mutated in cancer. Thus, the exploitation of synthetic lethal interactions in cancer cells can significantly expand the number of oncology drug targets. Secondly, the effects of drugs that have no (or limited) clinical activity as single agents could be greatly be effective when used in combination with a second drug that is synthetic lethal with the first drug. For example, inherited loss-of-function mutations in *BRCA1* and *BRCA2* genes predispose to tumour of, primarily, the breast and ovaries. Because *BRCA* gene products have a role in homologous recombination (HR) during repair of double strand DNA breaks, it was hybothesized that inhibiting additional DNA repair system could be synthetic lethal with the loss of *BRCA* gene function (Farmer et al. 2005).

Certainly, inhibitors of the PARP enzyme which has roles in excision repair, were found to be strongly synthetic lethal with mutations in *BRCA1* and *BRCA2* genes. Following encouraging clinical studies in 2009 by Fong et al., the use of the PARP inhibitor olaparib was approved for treating *BRCA*-mutated ovarian cancer in late 2014. However, quite a few clinical trials are ongoing that take advantage of synthetic lethal

interactions between BRAF and EGFR inhibitors identified in the laboratory including National Trial numbers 01719380, 01750918, and 01791309. There are also multiple trials based on the synthetic lethal interaction between MEK inhibitors and panHER inhibitors in KRAS-mutant cancers, including National Clinical Trial numbers 02039336, 02230553, and 02450656 (Bernards 2012).

Another example where the function of the *p53* tumor suppressor protein is lost in almost all cancers, either through mutation of the *TP53* gene or by alterations in components that control p53 activity. Given the role of *p53* in response to cellular stress, DNA damage, and DNA repair, the loss of *p53* creates vulnerabilities that can be explored as targets in the context of synthetic lethality. Synthetic lethal interactions have been found between *p53* and ATM/CHK2 (inhibitor/kinases respectively) pathway to maintain the genomic integrity and DNA damage response (Fedier et al. 2003).

Similar to *TP53*, the *RB1* pathway is inactivated in most of the human cancers, either through mutations of the *RB1* gene or by alterations in the pathways that control *pRB* activation including the loss of expression (encoding *p16*), overexpression or amplification of D-type cyclins. , a number of synthetic lethal interactions have been found between RB1and .

Hence, once we understand the sequence by which new vulnerabilities arise in cancer, we may be able to treat cancer sequentially with therapies that are at least as effective as, or even more effective than the first-line therapy.

1.6 CANNABINOID RECEPTOR 1 GENE (*CNR1*) AS A POTENTIAL TSG IN PROSTATE CANCER

1.6.1 THE HISTORY OF CANNABINOID DISCOVERY:

Human beings have used cannabis for thousands of years. The first archeological discovery is from China around 4000 BC. In 2737 BC, Shen Nung, the Emperor of China, was the first known to describe the properties and therapeutic potential of cannabis (Ware, Doyle et al. 2003). From the hemp plant *Cannabis sativa* more than 400 different chemicals can be extracted, and more than 80 are grouped under the name cannabinoids or phytocannabinoids. The main psychoactive compound is Δ^9-tetrahydrocannabinol (THC). In 1964 the chemical structure of THC was reported, and in 1988, the first demonstration of cannabinoid binding sites in the rat brain was published (Gaoni and Mechoulam 1964, Devane, Dysarz et al. 1988). After the discovery of the binding sites, Matsuda and coworkers published the DNA encoding a G protein-coupled receptor, which was activated by the cannabinoids and thereby called cannabinoid receptor type 1 (CNR1) (Matsuda, Lolait et al. 1990). Later, a second G-protein coupled cannabinoid receptor, cannabinoid receptor type 2 (CNR2), was cloned (Munro, Thomas et al. 1993). In addition, other receptors, including the transient receptor potential cation channel subfamily V member 1 (*TRPV1*) and certain orphan G protein- coupled receptors, *GPR55*, *GPR119* and *GPR18*, have been proposed to act as endocannabinoid receptors (Pertwee, Howlett et al. 2010).

There are three types of cannabinoids (Figure 1.7), plant-derived cannabinoids such as THC, synthetic cannabinoids such as WIN-55-212-2, JWH-13 and methanandamide (MET) and endogenous cannabinoids also known as endocannabinoids such as anandamide (ANA) and 2-Arachidonoylglycerol (2-AG) that are produced in the human bodies. The cloning of the receptors led to the identification of the endogenous ligands, called endocannabinoids. The first to be discovered was AEA (Devane, Hanus et al. 1992) and thereafter 2-AG (Mechoulam, Ben-Shabat et al.

1995). Other substances have been proposed to be endocannabinoids, such as 2- arachidonyl-glycerol ether (noladin ether) (Hanus, Abu-Lafi et al. 2001), and virodhamine (Porter, Sauer et al. 2002) but these have not yet been formally accepted as endocannabinoids. These derivatives have drawn renewed attention because of their diverse pharmacologic activities such as growth inhibition, anti-inflammatory effects, tumour regression, targeting angiogenesis and cell migration (Sarfaraz, Afaq et al. 2006, Alexander, Smith et al. 2009, Díaz-Laviada 2011, Guindon and Hohmann 2011, Velasco, Sanchez et al. 2012).

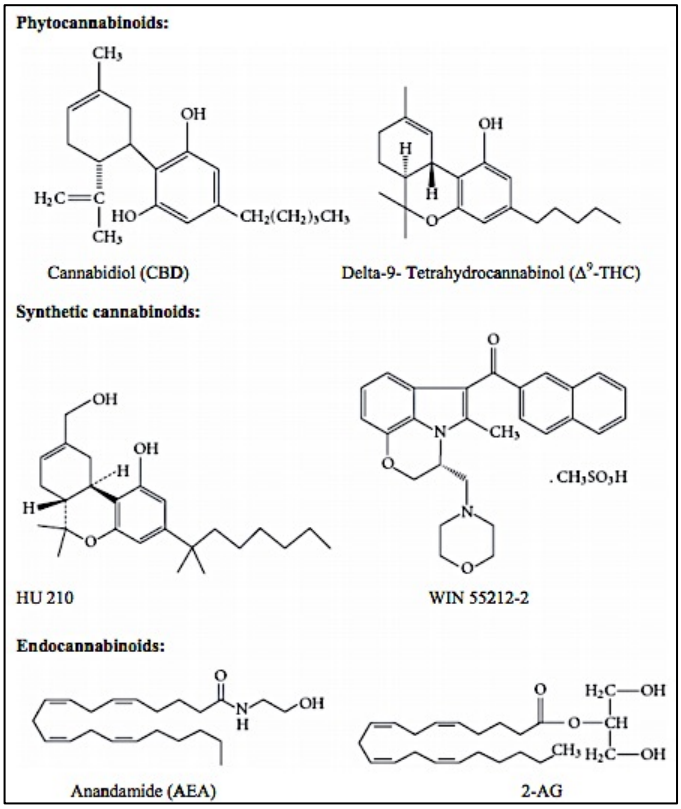


Figure 1.7 <u>The chemical structure of some cannabinoids</u>. There are three types of cannabinoids, the phytocannabinoids that are derived from marijuana, synthetic cannabinoids and the endogenous cannabinoids. Adapted and modified from The Endocannabinoid system (Javid, Phillips et al. 2016).

The endocannabinoid system is a neuromodulatory system comprised by endogenous ligands (N-anachidonoylethonalamine, AEA, anandaminde,

and 2-arachidonoylglycerol, 2-AG), which are lipid molecules that generated in the cell membrane from phospholipid precursors (Figure 1.8). These endocannabinoids are synthesized on demand at the site of action in response to specific signals, such as an increase intracellular calcium or activation of phospholipase C-**β** by $G_{q/11}$ metatropic receptors (Basavarajappa, Yalamanchili et al. 2008). Degradation of the endocannabinoids occurs locally by fatty-acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Hermanson and Marnett 2011) (Marnett et al 2011). It has been reported that the uptake of endocannabinoids from the extracellular space occurs by facilitated diffusion mediated by selective transporter (Fowler 2013).

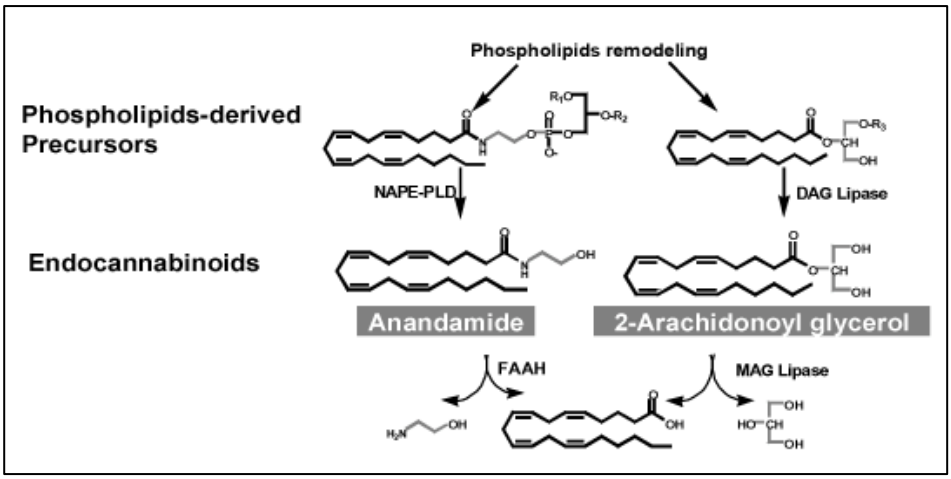


Figure 1.8 Endocannabinoids are long chain polyunsaturated fatty acid. Anandamide and 2-arachidonoyl glycerol (2-AG) are produced from the phospholipids through pathways that use NAPE-PLD (N-acylphosphatidylethanolamine-selective phospholipase D) and DAG (Diacylglycerol) lipase synthesis enzymes. They rapidly metabolized and hydrolyzed by FAAH (Fatty Acid Amide Hydrolase) and MAGL (Monoacyl lipase) enzymes. Adapted from (Di Marzo, Melck et al.).

The specific cannabinoid receptors are expressed in numerous cell types in the body and modulate many biological functions. In the central nerve system (CNS) the endocannabinoid system participates in the control of motor coordination, memory, learning, appetite and pain. Most neural functions controlled by endocannabinoid signalling depend on the neural

CNR1). This receptor is the main mediator of the inhibition of neurotransmission by retrograde signalling mediated by endocannabinoids (Hermanson and Marnett 2011).

1.6.2 CANNABINOID RECEPTOR 1

Cannabinoid receptor type 1 (CNR1), also named CB1, is a member of the G protein-coupled receptor superfamily possessing seven transmembrane domains with 68% amino acid homology within the transmembrane domains (Munro, Thomas et al. 1993). It is highly expressed in brain regions such as hippocampus, basal ganglia, and cerebellum (Demuth and Molleman 2006) and also expressed in peripheral tissues including eye, spleen, leucocytes and uterus, prostate (Bifulco, Laezza et al. 2006), and skeletal muscle (Eckardt, Sell et al. 2009). Further studies have shown that CNR1 is mainly expressed presynaptically on axon terminals (Katona *et al.*, 2001). The distribution of the cannabinoid receptors appears well conserved between many vertebrate species.

The human *CNR1* gene (*hCNR1, CB1*) is located on chromosome 6 locus q15 (Onaivi, Leonard et al. 2002) Figure 1.9A. Exon 4 contains the entire protein coding regions of human *CNR1*, while the three non-coding exons, named exon 1, 2, and 3 are located 5' to the protein-coding region and are separated by three introns (introns 1, 2, and 3) (Zhang, Ishiguro et al. 2004). Alternative splicing of the 5'-UTR of the *hCNR1* gene results in the formation of six *hCNR1* transcripts with variable 5' UTR, termed variants 1, 2, 3, 4, 5 and 6 (Figure 1.9B). Each of these variants has a unique 5'-UTR, transcription initiation site and distribution pattern in the human brain and peripheral tissues (Miller and Devi 2011).

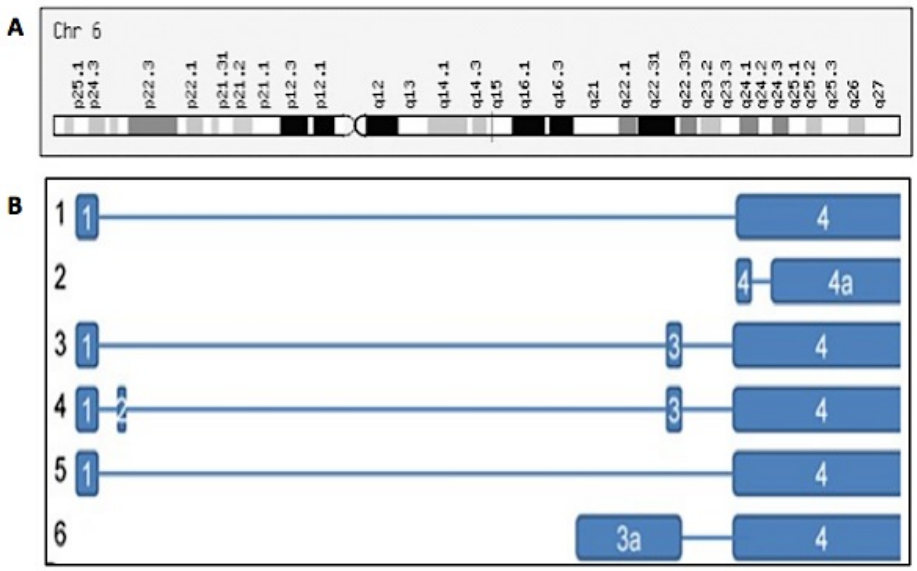


Figure 1.9 (A) The CNR1 genomic location: locus according to *Ensemble.* (B) Schematic diagram of the human *CNR1* gene and mRNA variants. Six splice variants of the 5'UTR of *hCNR1 (hCB1)* gene have been identified. Blue Boxes indicate exons, while interconnecting blue lines indicate introns. The coding region is located in exon 4, while the non-coding regions are exon 1,2 and 3. Adapted and modified from (Zhang, Ishiguro et al. 2004, Laprairie, Kelly et al. 2012)

The translation of the hCNR1 starts at the first ATG located at the 5' end of un-spliced exon 4 and produces a polypeptide chain of 472 amino acids. This chain forms an exceptionally long extracellular N-terminal tail of 116 amino acids connected to seven transmembrane domains and ended by an intracellular carboxyl terminus (The National Centre for Biotechnology Information, NCBI;(Zhang, Ishiguro et al. 2004) (Figure 1.10). Alternative splicing of *hCNR1* within the coding region has been identified, hCNR1a (411 amino acids; (Shire, Carillon et al. 1995, Ryberg, Vu et al. 2005). Subsequently, the second hCNR1 splice variant mRNA, hCNR1b (439 amino acids) has been identified (Shire, Carillon et al. 1995, Ryberg, Vu et al. 2005). The *hCNR1a* transcript lacks an internal segment of 167 base pairs within the sequence encoding the N-terminal tail of the receptor. Translation of the hCNR1a starts at the second ATG located at the 5' end of exon 4. The resulting receptor is shorter than hCNR1 by 61 amino acids at its N-terminus. In addition, the first 28 amino acids of the N-terminus are

totally different to hCNR1, while the remaining 27 amino acids are similar to the hCNR1. hCNR1a also lacks two out of three glycosylation sites and resulted in a more hydrophobic receptor. *hCNR1b* transcript is missing an internal segment of 99 base pairs resulting in a protein lacking 33 amino acids at the N-terminus tail. However, unlike hCNR1a, translation of hCNR1b starts at the first ATG located in exon 4 as hCNR1(Shire, Carillon et al. 1995, Zhang, Ishiguro et al. 2004, Ryberg, Vu et al. 2005) (Figure 1.10& 1.11).

The existence of these isomers on hCNR1 has raised questions concerning their functional variation. One of the reports characterized the hCNR1a splice variant and found that the binding of THC and WIN5521-2 agonists was slightly higher than binding to hCNR1 or hCNR1b, when the isoforms were stably expressed in Chinese hamster Ovary (CHO) cells (Rinaldi-Carmona, Calandra et al. 1996). Activation of hCNR1a by CNR1 agonists was able to inhibit cAMP and increase MAP kinase phosphorylation compare to the full-length hCNR1. In contrast, it has been reported that *hCNR1a* and *hCNR1b* mRNAs are translated and expressed as functional receptors *in vivo*. They found that hCNR1; hCNR1a were expressed at a similar level. However, hCNR1b were expressed at 2.5-fold higher level than hCNR1 in HEK293 cells (Ryberg, Vu et al. 2005). In addition, they found that all the hCNR1 were expressed to a similar degree at the membrane in HEK293 cells however, hCNR1a and hCNR1b displayed less affinity when they treated with the endogenous cannabinoid ligands (anandamide) (Ryberg, Vu et al. 2005). A more recent study published by Straiker's team, that hCNR1 variants were all robustly expressed in cultured hippocampal neurons (Straiker, Wager-Miller et al. 2012). Given difference in general studies, further investigation is required to understand the signalling properties of the hCNR1 variants.

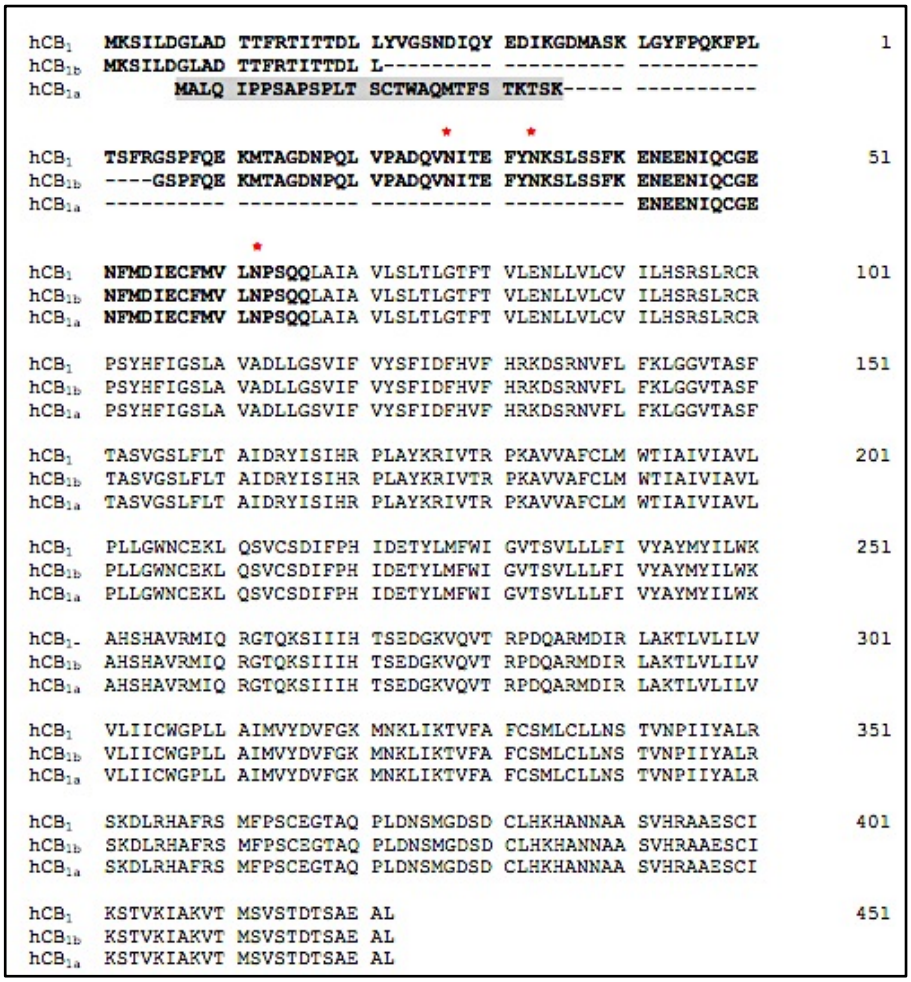


Figure 1.10 Amino acids sequence alignment of the human CNR1 and its splice variants CNR1a (CB1a) and CNR1b (CB1b), Modified from Kofalvi 2008. N-terminus of the hCNR1 consists of 116 amino acids, indicated in bold. The hCNR1a uses a different initiation coding leading to a frameshift from the reading frame of hCNR1, with only 55 amino acids N-terminal tail that differs from hCNR1 in the first 28 amino acids (highlighted in gray). The hCNR1b lacks an internal segment of 33 amino acids in a N-terminal tail. Glycosylation sites are indicated in red, - represent missing nucleotides. Adapted and modified from Laprairie, Kelly et al. 2012.

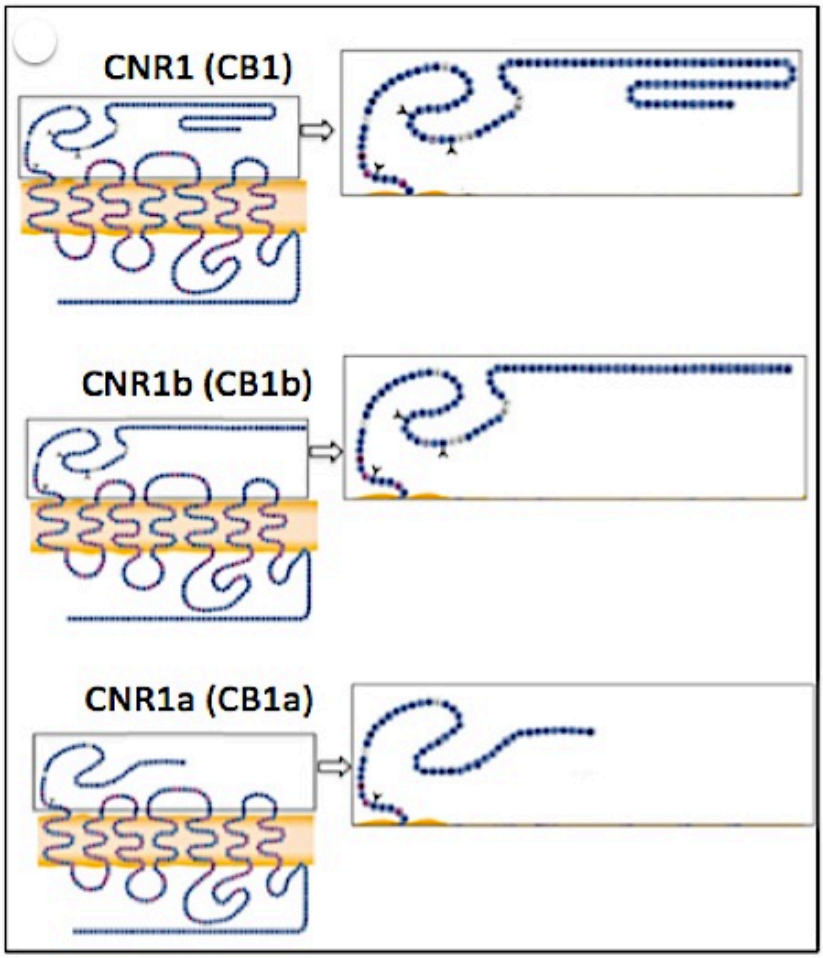


Figure 1.11 Schematic illustration of the amino acid sequences of hCNR1 (hCB_1), hCNR1b (hCB_{1b}) and hCNR1a (hCB_{1a}). adapted and modified from Cayman chemical company (https://www.caymanchem.com/Home).

1.6.3 CANNABINOID RECEPTORS SIGNALING PATHWAYS

The majority of CNR receptors are coupled to G proteins, mostly of the $G_{i/o}$ type. As a consequence of this special coupling, activation of cannabinoid receptors, CNR1 and CNR2 primarily leads to the inhibition of adenylyl cyclase and reductions in cyclic AMP accumulation in most tissues and models. The major mediators of CNR1 are the G proteins of the $G_{i/o}$ family (Turu and Hunyady 2010), which inhibit adenylyl cyclases in most tissues and cells, and regulate ion channels, including calcium and potassium ion channels (Demuth and Molleman 2006, Basavarajappa, Yalamanchili et

al. 2008, Guindon and Hohmann 2011, Straiker, Wager-Miller et al. 2012).

CNRs produce a number of different intracellular signaling responses that are dependent upon the cell type and situation investigated. One of the signaling pathways is Retrograde signaling in the brain. In glutamatergic synapses, the release of glutamate results in activation of postsynaptic ionotropic glutamate and the endocannabinoid system is not engaged. However, excessive glutamate signaling leads to activation of metabotropic receptors that are localized postsynaptically. Activation of these receptors leads to the synthesis of endocannabinoids, which then diffuse back across the synapse and bind to CNR_1 receptors on presynaptic terminals of neurons, where they inhibit release of glutamate. This is achieved as a result of the inhibition of voltage-activated Ca^{2+} channels, and activation of inwardly rectifying K^+ channel (Castillo, Younts et al. 2012). GABAergic neurons are also modulated by endocannabinoids, and retrograde endocannabinoid signaling has been demonstrated to be involved in electrophysiological processes such as depolarization-induced suppression of excitation, depolarization-induced suppression of inhibition, and long-term potentiation. In most cases, the use of selective inhibitors of endocannabinoid inhibition and/or knockout mouse models have indicated that 2-AG is the main endocannabinoid involved in these processes (Castillo, Younts et al. 2012)

Furthermore, cannabinoid receptors have also been shown to modulate other several signalling pathways that are more directly involved in the control of cell proliferation and survival, including extracellular signal-regulated kinase (ERK) (Bouaboula et al., 1995), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) (Liu et al., 2000; Rueda et al., 2000), phosphatidylinositol 3-kinase (PI3K)/Akt (Go´mez del Pulgar et al., 2000), focal adhesion kinase (Derkinderen et al., 1996), and maybe the ceramide pathway (Guzma´n et al., 2001) (Figure 1.12).

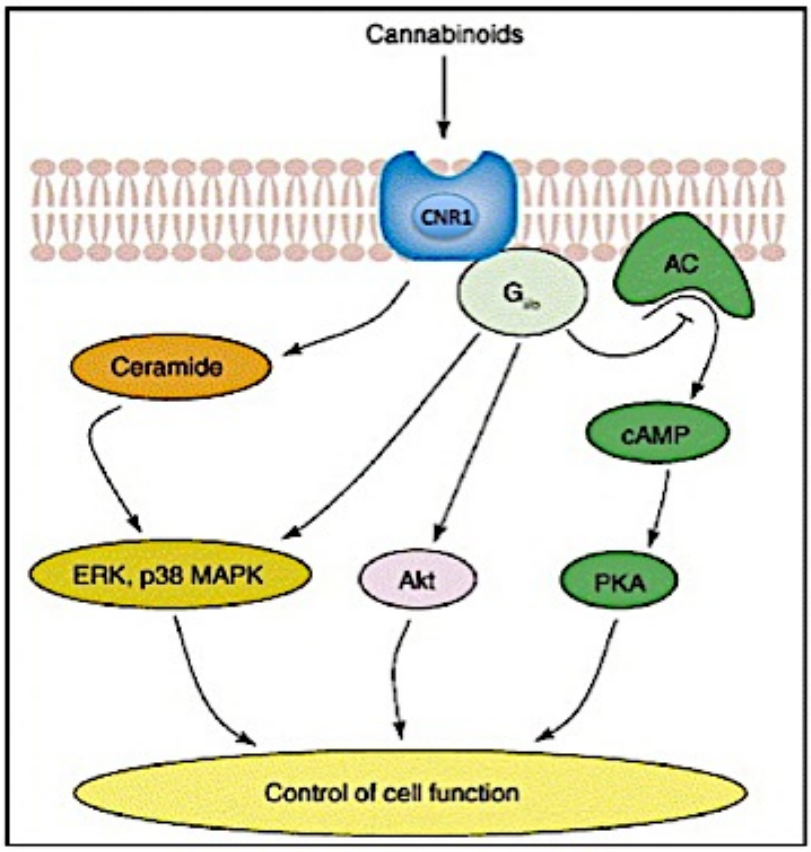


Figure 1.12 Signalling pathways coupled to the CNR1 (CB1). Cannabinoids bind to specific receptors. The $G_{i/o}$-protein-coupled CNR1 (CB_1) receptor signals to several different cellular pathways, including the adenylyl cyclase (AC), cAMP and protein kinase A (PKA) pathway; MAPK cascades (ERK, p38 MAPK); the Akt pathway; and a pathway for *de novo* synthesis of ceramide.

1.6.4 CANNABINOID RECEPTOR 1 AND PROSTATE CANCER

In a healthy prostate, *CNR1* is expressed in two regions- the parasympathetic nerves where it modulates contraction of the prostate duct and in the epithelial cells of the prostate duct, where it regulate prostate secretion (Díaz-Laviada 2011). Other reports described the immunolocalization of *CNR1* is primarily found within the epithelial lining of the prostatic duct, epithelial grandular and neuroendocrine cells with little or no staining of the stroma (Ruiz-Llorente, Sanchez et al. 2003, Chung, Hammarsten et al. 2009, Czifra, Varga et al. 2009). These findings

indicate that *CNR1* is highly expressed in the prostate epithelium. *CNR1* have been localised in the human prostate as well as in the biopsy from patients with BPH and prostate cancer

There is evidence indicating that cannabinoid receptors (*CNR1* and *CNR2*) could be an important target for the treatment of prostate cancer (Idris 2012, Van Dross, Soliman et al. 2013). Several studies have now evaluated the expression of *CNR1* in different prostate cancer tissue/cell lines (Snchez, Snchez et al. 2003, Sarfaraz, Afaq et al. 2005, Sarfaraz, Afaq et al. 2006, Olea-Herrero, Vara et al. 2009, Sharma, Hudson et al. 2014). Previous exon array analysis in our group showed that the cannabinoid receptor 1 (*CNR1*) gene, located in 6q15, was downregulated in all prostate cancer cell lines and clinical prostate cancer samples at the mRNA level compared to normal tissue (Shan 2010). Our group finding was supported by other reports published such as the expression of *CNR1* was down-regulated in human colorectal cancer (CRC) due to methylation of its promoter (Wang, Wang et al. 2008); mutation in the coding exon of CNR1 receptors was detected in oesophegal and colorectal cancers (Bedoya, Meneu et al. 2009, Bedoya, Rubio et al. 2009, Larrinaga, Begoaa et al. 2010, Meneu-Diaz, Bedoya et al. 2011); and CNR1 receptor was found to be down-regulated in Clear Cell Renal cell carcinoma using RT--PCR and western blot (Larrinaga, Begoaa et al. 2010).

Nevertheless, different observation also supported that, CNR1 was upregulated in prostatic adenocarcinoma tissues, and several cell lines including PC-3, DU-145, LNCaP, CWR22Rv1 and CA-HPV-10, CNR1 and CNR2 expression levels were higher, as compared with normal prostate epithelial cells (Sarfaraz, Afaq et al. 2005, Chung, Hammarsten et al. 2009, Czifra, Varga et al. 2009, Brown, Cascio et al. 2010, Sharma, Hudson et al. 2014). These observations agreed with my findings where CNR1 was upregulated in prostate cancer cell lines including DU145, LNCaP, and 22RV1. Furthermore, an extensive study of 399 human prostate cancer samples revealed the expression level of CNR1 was considerably higher in prostate cancer tissues than in normal prostate tissues (Chung, Hammarsten et al. 2009). Other cancer types including

hepatocellular carcinoma (Xu, Liu et al. 2006) and pancreatic ductal adenocarcinoma showed an increased level of CNR1 expression in human pancreatic tumour cell lines as well as in biopsies of human pancreatic tumours, whereas in samples obtained from normal pancreatic tissue, mRNA levels for these receptors were very low or could not be detected (Carracedo, Gironella et al. 2006)

Relatively large amount of data have accumulated during the last decade about the role of CNR1 receptors in tumour generation and progression. In many cases, these reports showed that levels of CNR1 are increased in cancers including prostate cancer, a situation that frequently correlates with tumour aggressiveness (Malfitano, Ciaglia et al. 2011)

In prostate cancer, it was found that patients with a tumour with higher CNR1 expression had a significantly higher proportion of Gleason scores 8–10, and metastases at diagnosis (Chung, Hammarsten et al. 2009). For 269 cases, tumour CNR1 was measured for patients who only received palliative therapy at the end stages of the disease, allowing the influence of CNR1 upon the disease outcome to be determined. CNR1 in non-malignant tissue was not associated with disease outcome. A tumour CNR1 score $\geqslant 2$ was associated with a significantly lower disease-specific survival indicating that a high tumour CNR1 score is associated with prostate cancer severity of the disease and poor prognosis (Chung, Hammarsten et al. 2009). In other types of cancer, Fowler et al, reported that the level of CNR1 receptor expression in colorectal cancer is associated with the tumour grade in a manner dependent upon the degree of CpG hypermethylation. They found that the high CNR1 is indicative of a poorer prognosis in stage II microsatellite stable tumour patients (Gustafsson, Palmqvist et al. 2011). Furthermore, CNR1 receptor levels are also increased and correlate with disease severity in human epithelial ovarian tumours (Messalli, Grauso et al. 2014) and have been proposed to be a factor of bad prognosis following surgery in stage VI colorectal cancer (Jung, Kang et al. 2013, Velasco, Hernández-Tiedra et al. 2015).

Despite the above-discussed conflicting data relative to the role of CNR1 receptors in tumour progression, during the last two decades many reports have shown that in experimental models of different types of cancer, cannabinoid receptor agonists exerted antitumor effects.

In prostate cancer, the treatment with cannabinoid receptor agonists, including R (+)-Methanandamide (MET) and WIN-55, 212-2, resulted in inhibition of cell growth in LNCaP and PC3 cells, and this was associated with inhibition of AKT and activation of ERK (Snchez, Snchez et al. 2003, Sarfaraz, Afaq et al. 2005, Sarfaraz, Afaq et al. 2006, Olea-Herrero, Vara et al. 2009). Data also showed that treatment of LNCaP prostate cancer cells with WIN-55, 212-2 (*CNR1/2* agonist) results in a significant dose- and time-dependent decrease in cell viability and increased apoptosis with an arrest of the cells in the G0-G1 phase of the cell cycle; induction of p53 and p27/KIP1 genes; down-regulation of cyclins D1, D2, E; decrease in the expression of cdk-2, -4, and -6 (Sarfaraz, Afaq et al. 2006). Also, cannabinoid receptor inhibition of cell proliferation and invasion has also been associated with induction of p27kip and inhibition of cell cycle regulatory molecules, CDKs, and inhibition of VEGF expression in androgen-dependent cancer cells has been also reported (Bifulco, Laezza et al. 2006).

The anti-proliferative and apoptotic effects of the endogenous cannabinoid ANA in LNCaP, DU145, and PC3 were also reported been mediated through down-regulation of epidermal growth factor receptor (EGFR) and accumulation of ceramide (Mimeault, Pommery et al. 2003). Interestingly, the ANA analogue (R)- methanandamide was shown to have a mitogenic effect on LNCaP cells at very low doses (Snchez, Snchez et al. 2003). Apart from the inhibition of cell growth, the ligand of *CNR1*, endogenous 2-arachidonoylglycerol (2-AG), has also been reported to inhibit invasion of prostate cancer cells (Ramer and Hinz 2008).

Several studies have evaluated the antitumor effect of CNR1 receptors in different cancers tissue/cell lines. For instance, in breast cancer, Anandamide (AEA) is important lipid ligands regulating cell proliferation, differentiation and apoptosis. It was reported that anandamide inhibits basal and nerve growth factor (NGF) induced proliferation of MCF-7 and EFM-19 cells in culture through CNR1 receptor and Δ^9-THC inhibits 17beta-estradiol-induced proliferation of MCF7 and MCF7-AR1 cells (Melck, Petrocellis et al. 2000). Cell death by apoptosis is the result of cell cycle arrest. The analogue of anandamide, Met-F-AEA reduces MDA-MB-231 proliferation by arresting cells in the S phase of the cell cycle (Grimaldi, Pisanti et al. 2006). Furthermore, treating NSCLC cell lines (A549 and SW-1573) with CNR1/CNR2- agonists Win55, 212-2 significantly attenuated the growth of cells. Also observed a significant reduction in focal adhesion complex, which plays an important role in migration, upon treatment with Win55, 212-2. In addition, CNR1 agonists Win55, 212-2, significantly inhibited *in vivo* tumour growth and lung metastasis (~50%). These effects were receptor mediated, as pre-treatment with CNR1/CNR2 antagonists abrogated CNR1/CNR2 agonist–mediated effects on tumour growth and metastasis (Preet, Qamri et al. 2011).

1.7 PREVIOUS WORK IN OUR TEAM AND RATIONALE FOR MY THESIS PROJECT

In a previous study by Shan 2010, FISH analysis was performed on 68 prostate cancer samples and six morphologically non-malignant prostate samples (Shan 2010). Thirteen of 28 (out of 68) prostate cancer samples (46%) were assessed as 6q15-deletion positive. The genes downregulated in the deletion region were examined using our Exon Array (Affymetrix Exon 1.0 ST) data of six prostate cancer cell lines (LNCaP, PC3, 22RV1, DU145, VCaP and MDA PCa 2b), one clinical prostate cancer sample (p127) and two BPH samples (p143 and p2) as a control. *CNR1* on 6q15 was down-regulated in the six prostate cancer cell lines and one clinical prostate cancer sample. qRT-PCR analysis was applied to validate the down-regulation of this gene. Relative expression of *CNR1* gene measured by qRT-PCR was consistent with the Exon Array data, showing down-regulation in all prostate cancer cell lines and the clinical sample (p127) compared to the BPH samples. Followed by qRT-PCR validation of *CNR1* gene in enlarged clinical prostate cancer samples (BPH and tumour samples), *CNR1* was still found down-regulated in these samples. However, detection of the CNR1 protein failed. Although the antibody (Ab23703, Abcam) was demonstrated to be specific for immunohistochemistry IHC using BPH as a positive control, in western blot analysis using the same antibody was inconclusive.

In contrast, several studies have shown that the CNR1 was highly expressed in prostate cancer cell lines at protein levels using western blot analysis (Sarfaraz, Afaq et al. 2005, Czifra, Varga et al. 2009). Although it is possible that the differences generated between the studies were caused by different cell linages or cell culture conditions, such as different media and growth factors, the misrecognition of a nonspecific band at the expected size of the CNR1 protein might have been the main reasons for these conflicting results. The conflict between a potential tumour suppressor role and reported high expression of *CNR1* may by due to the difficulty in accurate detection of CNR1 protein expression caused by the uncertainty of a specific and reliable antibody. Moreover, as *CNR1* is

located at 6q15, one of the most frequently deleted regions in prostate cancer; DNA mutation or methylation may reduce the receptor activity. Also, deregulation in downstream signalling pathways in some of the patient samples may have caused the reduction of receptor activity.

In light of this and the previous findings in our laboratory, an investigation was taken into the hypothesis that *CNR1* is a prostate cancer tumour suppressor.

1.8 AIMS OF THIS THESIS

I aimed to investigate whether *CNR1* could act as a TSG in prostate cancer by characterising its status in prostate cancer cell lines and clinical samples and investigating its functional roles.

The objectives of this project were:

- Investigate the expression of CNR1 in prostate cancer cell lines (22RV1, LNCaP, DU145, PC3, and VCaP) and immortalised prostate epithelial cell lines (PNT1a and PNT2) at mRNA and protein levels
- Evaluate the antibodies to select the most reliable one for further determination of CNR1 expression levels, using different approaches for CNR1 knockdown by siRNA silencing and glycosylation inhibitors
- Identify any mutations in the *CNR1* gene using Next Generation Sequencing analysis and if genomic polymorphism in the gene associated with prostate cancer risk. Analyse the function of *CNR1* by overexpression and knockdown of this gene in relevant prostate (cancer) cell lines using MTS assay, colony formation assay and migration and invasion assays
- Determine the therapeutic potential of cannabinoid analogues such as HU210 in treating prostate cancer and using CNR1 protein expression as a biomarker

2 MATERIAL AND METHODS

2.1 CELL LINES

Five prostate cancer cell lines, including 22RV1, LNCaP, DU145, PC3 and VCaP (obtained from ATCC), two SV40-immortalized prostate epithelial cell lines PNT1a and PNT2, (obtained from Norman Maitland and Colin Cooper, respectively), and normal prostate epithelial cells PrEC (obtained from Lonza), were selected for this study. All cells were tested and authenticated in August 2010, using the ABI AmpF/STR identifier kit. Table 4 describes the cell type, PSA and AR status of each cell line.

Table 2.1 Human prostate cell lines

Cell line	Cell type	PSA status	AR status
22RV1	Human prostate carcinoma epithelial	pos	wt/mt active
LNCaP	Human prostate carcinoma	pos	mt active
DU145	Human prostate carcinoma	neg	neg
PC3	Human prostate carcinoma	neg	neg
PNT1a	Immortalized prostate epithelia	wt	wt
PNT2	Immortalized prostate epithelia	wt	wt
PrEC	Normal prostate epithelia	wt	wt

AR, and PSA expression status: pos: positive; neg: negative; wt: wild type; mt: mutant.

2.2 CELL CULTURE

All cells were stored in liquid nitrogen in 10% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich), 20% Foetal Calf Serum (FCS) (Life Technology), and 70% RPMI (Cancer Research UK) or Dulbecco's DMEM media (Cancer Research UK). All cell lines were cultured in RPMI or DMEM media supplemented with 10% FCS and 1% Penicillin/Streptomycin (100 units/ml). Cells were then pelleted by centrifugation at 1200 rpm for 5 min, the supernatant was discarded and the cell pellet was re-suspended in 5 mL of culture medium. Cell suspensions were maintained in T75 culture flasks and incubated at 37°C in humidified atmosphere with 5% CO_2.

The cells were grown in tissue culture flasks and the media renewed every 72 h of incubation. When cells were 80% confluent, they were sub-cultured by adding 1x Trypsin-Ethylenediaminetetraacetic Acid (Trypsin-EDTA) into the flask without the culture medium and incubating at 37°C for 5 min. Next, 5 ml RPMI/10%FCS media was added to the flasks to deactivate the trypsin. Cell suspensions were then collected in 50ml tubes and centrifuged at 1200 rpm for 5 min. The supernatants were decanted and the cell pellet was re-suspended in pre-warmed fresh 10% FCS media, transferred to new flasks and incubated at 37°C with 5% CO_2 for further culture.

2.3 TISSUES AND BLOOD SAMPLES

2.3.1 Formalin-fixed paraffin embedded (FFPE) clinical samples

73 prostate cancer samples were collected from Bart the London hospital by the Orchid tissue bank. All samples were formalin fixed and paraffin embedded after removed from the patients. The collection of these specimens was approved by the Local Ethical Committee.

2.3.2 Blood samples from prostate cancer patients

13 blood samples from prostate cancer patients were collected from Bart the London hospital. 96 blood samples taken from Chinese non-cancer prostate patients and 107 blood samples taken from Chinese prostate cancer patients. The blood samples from prostate cancer patients were obtained from shanghai hospital. Each sample was frozen and stored at -80C until analysis was done. The collection of these specimens was approved by Local Ethical Committee

2.4 CNR1 AGONIST (HU210) AND ANTAGONIST R-SLV- TREATMENT

CNR1 agonist HU210 was purchased from Tocris Bioscience, UK. CNR1 antagonist (R)-SLV-319 was purchased from Cayman Chemicals, UK. Both, HU210 and (R)-SLV-319 were dissolved in DMSO at a stock concentration of 100 mM, aliquot and stored at -20°C until further use. For each experiment, required concentrations were freshly prepared by diluting the 100 mM stocks with an antibiotic/FBS-free medium.

Cells ($1x10^4$) were pre-seeded in 6-well plates and incubated in penicillin-free, 10% FBS RPMI media under standard conditions. After 24 h incubation, cells were treated with different concentrations of HU210, (R)-SLV-319 or DMSO.

2.5 TUNICAMYCIN TREATMENT

Tunicamycin was purchased from Sigma-Aldrich. It was dissolved in DMSO at a stock concentration of 10 mg/ml, and diluted to the required final concentration with the antibiotic/FBS-free medium. It was stored at 4°C. Tunicamycin was used as a vehicle for different time points.

LNCaP cells were pre-seeded in 6-well plates and incubated penicillin-free 10% FBS under standard conditions. After 24 h incubation, cells were treated with 5 μM tunicamycin or DMSO for the inhibition of glycosylation

of CNR1 protein

2.6 RNA EXPRESSION ANALYSIS

2.6.1 TOTAL RNA EXTRACTION FROM CELL LINES

RNA was extracted using the Trizol (Invitrogen) according to the manufacturer's protocol. Briefly, 70% confluent cells were washed twice with PBS. Then, 1 ml of Trizol reagent was added to the cells and the homogenate was kept at room temperature for 1 min to ensure a complete dissociation of nucleoprotein complexes. Homogenate was transferred to a 1.5 ml tube and 200 µl of chloroform was added with vigorous shaking for 15 s. After incubating the mixture at room temperature for 5 min, the tube was spun at 12000 rpm for 15 min at 4°C. For RNA precipitation, the aqueous phase (upper phase) was transferred into a new 1.5 ml tube, mixed with 500 µl of isopropanol and incubated at room temperature for 10 min. The mixture was centrifuged at 12000 rpm for 15 min at 4°C, and the supernatant was decanted. To wash the RNA pellet, 500 ml of 75% ethanol were added to the pellet and the tube was centrifuge at 7500 rpm for 5 min at 4°C. The ethanol was carefully removed and the RNA pellet was air-dried for 30 min at room temperature, resuspended in 15-30 µl DEPC-treated water, incubated at 55°C for 10 min and cooled on ice immediately before storage at -80°C. Extracted RNA was quantified by using Nanodrop (Thermo Scientific) and quality determined by running 1% Agarose gel.

2.6.2 REMOVAL OF GENOMIC DNA FROM RNA SAMPLES

In order to remove any residual genomic DNA from the cell extracted RNA, DNase I (Ambion, Applied Biosystem) treatment was applied following the manufacturer's protocol. Briefly, 1 µl (2U) of DNase I was added for each 10 µg of RNA (in a 50 µl total volume solution with DEPC-treated water and DNase buffer). After incubation at 37 °C for 30 minutes, RNA was purified using standard phenol/chloroform extraction to inactivate the DNase I. Briefly, 100 µl of 1:1 phenol/chloroform was added to the sample, vortexed for 15 sec and centrifuged at 12000 rpm for 3 min. The top aqueous phase was transferred into a 1.5 mL fresh tube containing a mixture of 100 µl isopropanol and 5 µl 4M NaCl to precipitate RNA. To improve the RNA quality, RNA was precipitated by adding 250 µl of 100% ethanol and 10 µl of 3M NaOAc (pH 5.2, Invitrogen) to the samples. After 24 h incubation at -20°C, RNA samples were centrifuged at 12000 for 5 min at 4°C. The precipitated RNA was washed with 70% ethanol and air-dried at room temperature for 30 min. Finally, RNA was re-dissolved in DEPC-treated water and stored at -20°C until further use.

The yield of total RNA was determined by measuring the spectrophotometric absorption (optical density; OD) at 260 nm. The absorbance of RNA samples at 260 nm and 280 nm, diluted in sterile distilled water, was used to evaluate protein contamination (A_{260}/A_{280} OD ratio). The A_{260}/A_{280} OD ratio was determined and used to assess the purity of the sample (OD ratio = 1.8-2).

2.6.3 Synthesis of complementary DNA (cDNA)

The synthesis of cDNA was performed using M-MLV RT RNase H minus point mutant (Promega, UK). Briefly, 1 µg of total RNA was mixed with 1µl of 50 µM random hexamers and incubated at 70°C for 5 min. After cooling on ice, the sample was mixed with 6 µl of 5x synthesis buffer (Promega), 10 µl of dNTP (2.5mM each) and 2 µl M-MLV RT RNase H minus point mutant (200 U/µl). The reaction was then incubated at 42°C for 1 h followed by 95°C for 5 min. Finally, the cDNA samples were stored at -

20°C. A negative control (without DNA template) was always included to evaluate accidental reagent contamination.

2.6.4 cDNA CONFIRMATION USING B-ACTIN CONTROL POLYMERASE CHAIN REACTION (PCR)

Following the cDNA synthesis, a standard PCR using β-actin primers (Sigma) (Table 5). β-actin is a commonly used endogenous gene for PCR-based techniques and western blotting. Simply, β-actin was used as a positive control to determine the cDNA synthesised by checking for the presence or absence of PCR product and to ensure equal amounts of total cDNA in each sample. For each sample, the PCR reaction was performed in 25 µl volumes. A mixture of 1 µl of cDNA, 2.5 µl 10X PCR buffer (Invitrogen), 0.5 µl of dNTPs (10 mM), 0.75 µl of MgCl2 (50 mM, Invitrogen), 1 µl of each β-actin primer (10 µM each, Sigma), 0.2 µl of Taq DNA polymerase (5 U/µl, Invitrogen) and 17.05 µl of DEPC-treated water was used. The PCR was carried out under the following conditions: 95 ºC-1 min; 95 ºC-30 s, 62 ºC-30 s and 68 ºC-90 s for 35 cycles; 72 ºC for an additional 1 min after the final cycle. PCR products were analysed on a 1.5% agarose /Ethidium Bromide gel and bands of 250 bp were expected.

Table 2.2 Standard PCR using β-actin primers

Forward sequence	**5'-GCGGGAAATCGTGCGTGCGTGACATT-3'**
Reverse sequence	5'-GATGGAGTTGAAGGTAGTTTCGTG-3'

2.6.5 QUANTITATIVE REVERSE TRANSCRIPTION-PCR (QRT-PCR)

CNR1 gene expression was analysed using TaqMan qRT-PCR. TaqMan assays require a probe with a reporter and a quencher dye attached in addition to a pair of primers of the gene of interest. The probes are designed to bind to the sequence amplified by the primers and then the probe is cleaved by the Taq DNA polymerase during each cycle of the PCR reaction. Degradation of the probe releases the fluorophore from it

and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. This method was used as only the amplification of a specific target is detected; therefore the amount of *CNR1* DNA in each condition could be specifically quantified.

TaqMan gene expression probes (Table 2.3) were commercially obtained from Applied Biosystems, and qRT-PCR reactions were performed using an ABI 7500 Real-Time PCR system (Applied Biosystems) keeping the default settings for baselines and thresholds. Housekeeping gene *GAPDH* was used as an endogenous control. PCR reactions (25 µl) were prepared in triplicate on a 96-well plate. The reaction mix is listed in (Table 2.4) and the cycling conditions in (Table 2.5). In addition to the samples, a no-template control was included to look at DNA contamination of the samples and negative reverse transcriptase control was included to control for potential genomic DNA contamination in the RNA extraction.

Table 2.3 TaqMan specific probe/primer mix

Transcript	Assay ID	Interrogated sequence (Refseq)	Exon Boundary
CNR1	Hs00275634_m1	NM_016083.4*	1-2
GAPDH	Hs99999905_m1		3-3

*Homo sapiens cannabinoid receptor 1 (brain) (*CNR1*), transcript variant 1, mRNA

Table 2.4 TaqMan qRT-PCR reaction mixtures

Components	Volumes (µl)
2x Universal PCR Master Mix	12.50
20x ABI probe/primer mix	1.25

cDNA	2.00
Water	9.25

Table 2.5 QRT-PCR standard programs

Step	Temperature (°C)	Time
Initial denaturation	50	2 min
Denaturation	95	10 min
40 cycle		
Denaturation	95	15 sec
Annealing	60	1 min

Each probe reading generated an expression curve from which the cycle threshold (Ct) values were extracted and Ct mean was calculated from each triplicate. Each target mRNA was normalised against the endogenous control, GAPDH, by subtracting the Ct value of GAPDH from the Ct value of the target probe (ΔCt). To compare results against a control sample, the ΔCt for the control sample was subtracted from the ΔCt for the target sample (ΔΔCt). The fold change was compared with control was calculated by $2^{-\Delta\Delta Ct}$.

$\Delta Ct = \Delta C_{t\ target} - \Delta C_{t\ GAPDH}$

$\Delta\Delta C_t = (\Delta C_{t\ target} - \Delta C_{t\ GAPDH})_{sample} - (\Delta C_{t\ target} - \Delta C_{t\ GAPDH})_{control}$

Expression fold change = $2^{-\Delta\Delta Ct}$

2.7 PROTEIN EXPRESSION

2.7.1 TOTAL PROTEIN EXTRACTION OF CELL LYSATES

Media supplemented with 10% FBS was removed before cultured cells were washed with PBS buffer solution. 1 ml of lysis buffer (1% Triton X-100 (Sigma), phosphatase inhibitor and 1x protease inhibitor cocktail (Roche) in PBS) was applied to each well of a 6-well plate. Cells were scraped and pelleted in 4°C centrifuge at 2000 rpm for 2 min. The supernatant was stored at -80°C.

2.7.2 PROTEIN ASSAY

The method used to determine the protein concentration of the cell lysates was Bio-Rad's protein assay, which is based on the Bradford dye-binding procedure (Bradford 1976), and involves a colorimetric assay for measuring total protein concentration. Protein standards were prepared using Bovine Serum Albumin (BSA) (Sigma).

A standard curve of protein concentration was produced in duplicates using serial dilutions 0.1, 0.2, 0.3, 0.4, 0.5 and 1µg/µl. Protein was quantified by adding the whole cell extract (1:5) in DEPC-treated water, then 10 µl of the diluted samples was added into each well, then 190 µl of 1x BioRad solution (BioRad reagent (5x) diluted in H_2O) was added to the samples and to the bovine serum albumin protein standard. The 96-well plate was incubated at room temperature for 5 min and analysed using Opsys MR 96-well microplate reader with absorbance 595 nm. A standard

curve of absorbance versus concentration was constructed for the different concentrations of BSA and the protein concentration in each sample was calculated using the standard curve graph. Samples were stored at -80°C until further use.

2.7.3 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Protein samples (30-50µg) were subsequently incubated with 4X sample buffer, reducing buffer at 95°C for 5 min. Proteins of the whole cell lysate were separated on an SDS-PAGE (10-12%) by electrophoresis according to the apparent molecular weight for 120 min at 120 V. 20 µl of a molecular weight Rainbow marker was run in parallel to the samples in order to predict the size of the protein bands. The gel was transferred onto PVDF membrane using the Bio-Rad mini-PROTEIN Tetra gel electrophoresis system (BioRad Laboratories Ltd) for 100 min at 200A.

The membrane was re-activated with methanol and washed again in 0.2% TBS-tween before blocking for 24 h with 5% BSA or non-fat 5% milk (5% Marvel 0.2% Tween-20 in TBS) at 4°C. The membrane was washed in TBS-T three times for 10 min followed by incubation with the appropriate primary antibodies diluted in 5% BSA in TBS-T for 24 h at 4°C with agitation. The primary antibodies and dilutions used are shown in (Table 2.6). After the primary antibody incubations, the membranes were washed in TBS-T three times for 10 min each and incubated with horseradish peroxidase-conjugated secondary antibody for 45 min at room temperature on a plate shaker. Unbound antibody was removed by washing with TBS-T and the remaining immunecomplexes visualized using the enhanced chemiluminecence (ELC) western blotting detection system (Amersham Biosystem). Signal was visualized with RX Fuji X-Ray Film developed in a Curix 60 Developer. For loading control all blots were blocked with 5% BSA and re-probed with mouse anti-actin antibody using the same protocol described above.

Table 2.6 Antibodies and conditions

Protein	Source	Species	Western blot
hCNR1-CB1,	Abcam ab23703	Rabbit	1:100
hCNR1-CB1,	Santa Cruz, N-15 sc-10066	Goat	1:500
CNR1-L13	Prof Elphick pAb, 2824.3	Rabbit	1:500
Pro-Caspase-3	Cell signalling	Rabbit	1:1000
PARP and Cleaved (Asp214) PARP	Cell signalling	Rabbit	1:1000

2.8 IMMUNOFLUORESCENCE (IF)

Sterilised glass coverslips were placed in 12-well plates. $8x10^5$ cells were seeded on these coverslips, in 500 µl RPMI containing 10% FCS, and left to settle at 37°C for 24 h. Unbound cells were removed by washing with PBS and the remaining cells fixed at room temperature for 30 min using 4% formaldehyde. After two washes in PBS (5 min/each), the coverslips were treated with blocking solution (50% Donkey serum+ 0.1% soparin +PBS) for 30 min at room temperature. After two washes (5 min/each), the coverslips were incubated overnight with primary antibody at 4°C on a rotor. Afterward, coverslips were washed in PBS and incubated with the secondary antibody fluorescein-conjugated anti-rabbit IgG for 1 h at room temperature. After two washes in PBS (5 min/each), the coverslips were incubated with DAPI for 10 min at room temperature and then washed with PBS-T. The coverslips were mounted with fixation solution and observed by fluorescence microscopy.

2.9 FUNCTIONAL ASSAY

A pool of four siRNA was chosen based on highly conserved *CNR1* sequence (SMARTpool; Dharmacon Research) (Table 2.7). This combination was chosen in order to knock-down all *CNR1* isomers. BLAST searches were performed to verify the *CNR1* specificity. siRNA were diluted in the manufacturer's buffer (as recommended in Table 2.8). 10 nmoles of *CNR1* siRNA and non-targeting siRNA (NT siRNA) were dissolved in 1X siRNA buffer, which was diluted from 5X siRNA buffer (Dharmacon) in DEPC water, to a final concentration of 20 µM. The dissolved siRNA was incubated at room temperature for 30 min with rotation. The siRNA solution was stored in 50 µl aliquots at -20°C prior to use.

Table 2.7 siRNA target sequences

siRNA target	Name	Sequences
CNR1	siGENOME SMARTpool 1	CGGCAGUGAAGAACCGAUA
	siGENOME SMARTpool 2	CUGGAUGAGUAGCGCUAUA
	siGENOME SMARTpool 3	GCGAGAAACUGCAAUCUGU
	siGENOME SMARTpool 4	GGACAUAGAGUGUUUCAUG

Table 2.8 Recommended siRNA re-suspension volumes and concentrations

siRNA Amount (nMol)	siRNA Buffer to be added (mL)	Final siRNA concentration (µM)
10	0.50	20

20	1.00	20
50	1.00	20
100	1.00	100

2.9.1 TRANSFECTION WITH LIPOFECTAMINE 2000 REAGENT

One day before transfection, $5x10^5$ 22RV1 cells in 2 ml of growth media without antibiotics were plated in 6-well plates so that cells reached 90-95% confluence at the time of transfection. For each transfection, complexes were prepared as follows: 4 µl of DNA was diluted in 250 µl of serum-free/antibiotic-free growth media; 10 µl of Lipofectamine™ 2000 (Invitrogen) was diluted in 250 µl of serum-free/antibiotic-free growth media. After incubation at room temperature for 5 min, the diluted DNA was combined with diluted Lipofectamine™ 2000 followed by incubation at room temperature for 20 min. 250 µl of complexes were added to each well. Cells were incubated at 37°C in a CO_2 incubator for 24-48 h prior to testing for transgene expression.

2.9.2 REVERSE TRANSFECTION OF SIRNA USING OLIGOFECTAMINE

Knock-down studies were performed using DharmaFECT1 transfection reagents (Invitrogen) following the manufacturer's recommendations. In reverse siRNA transfection, siRNA, Oligofectamine reagents and cells were added to the wells at essentially the same time. $2.5x10^5$ cells in antibiotic/FBS-free were seeded in 6-well plates. For each well, 10 µl of 20 µM siRNA was diluted in 90 µl of 1X siRNA buffer plus 100 µl of antibiotic/FBS-free media, and 4 µl of Oligofectamine were diluted in 196 µl of antibiotic/FBS-free media. Both dilutions were incubated at room temperature for 10 min. Then, Oligofectamine mix was added to the siRNA and further incubated at room temperature for 25 min. Finally, cells with 1.6 ml of antibiotic-free media and 400 µl of siRNA mixture were added to

each well. Cells were harvested after 24, 48 and 72 h for RNA and protein quantification.

2.9.3 DOUBLE TRANSFECTION OF SIRNA USING OLIGOFECTAMINE REAGENT

Cells ($3x10^5$) were seeded per well in 6-well plates. Cells were cultured overnight in RPIM media supplemented with 10% FBS without antibiotics. 5µl of each *CNR1* siRNA or non-targeting (NT) siRNA were diluted in 180 µl DMEM. 5 µl of Oligofectamine (Invitrogen) was diluted in 20 µl of DMEM followed by incubation at room temperature for 10 minutes. 25 µl 25 µl of the Oligofectamine mix was added to the *CNR1* or NT siRNA mix and incubated at room temperature for 25 min. 210 µl of transfection mixtures were mixed with 790 µl of antibiotic/FBS-free DMEM. Cells in each well were then washed with PBS, and 1 ml of the transfection mixture with DMEM was added to each well. After 4 h, 500 µl of DMEM media, supplemented with 30% FBS, were added to each well. After 48 h post transfection, adherent cells were re-transfected with siRNA at the same concentration. Cells were incubated under standard conditions and harvested after 24, 48 and 72 h for protein and mRNA analysis.

2.9.4 MTS ASSAY

90 µl of suspended cells ($1x10^4$cells/well) in RPMI supplemented with 10% FCS were seeded in 96-well plates and incubated overnight under standard conditions. Next day, cell media was replaced with 100 µl of FCS-free media in each well. After suitable cell treatment, viability was assessed 1-day post-treatment (1 day after seeding) using the MTS viability assay following manufacturer's instructions (Promega, WI, USA). The tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) is converted by mitochondrial dehydrogenases in metabolically active cells to a soluble formazan compound, which absorbs light at 490 nm. The

product of the reaction is directly proportional to the relative number of live cells. After incubation at 37°C for 4-7 h, the plates were read using a microplate reader (Opsys MR, Dynex Technologies) at a wavelength of 495 nm. Values were corrected against the blank wells containing media without cells and expressed as percentage cell death compared to untreated control cells. Sigmoidal dose-response curves were generated by non-linear regression analysis using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego California, USA) to determine the effective concentration killing 50% of cells (EC_{50})-values for each agent or combination of agents in each cell line.

2.9.5 WOUND SCRATCH ASSAY

$1x10^6$ cells were seeded in 6-well plates and cultured in PRMI containing 10% FCS until a monolayer had formed. After suitable treatment, one single scratch was then made in the monolayer of each well using a 200 µl pipette tip. The media was removed and fresh RPMI media added. Images were taken of the scratch after 0 and 16 h using an Olympus light microscope at 40X magnification. The size of the wound was calculated in ImageJ software by analyzing the number of pixels the wound size contains, compared to the total area. Wound size represented as a percentage of the total area.

2.9.6 COLONY FORMATION ASSAY

Between 1000 and 500 of 22RV1 cells were seeded per well in a 6-well plate in RPMI media supplemented with 10% FCS and 1% penicillin. Cells were cultured for 7 days after suitable treatment. Culture media was changed every three days. The culture media was removed and colonies were washed twice with PBS. Colonies from each well were stained by adding 3 ml of 1% crystal violet (0.5 g dissolved in 500 ml of methanol) to the wells and incubating at room temperature for 20 min. Crystal violet was then aspirated and stained colonies were washed three times with distilled

water. The plate with colonies was left to air dry. Analysis took place by counting the number of colonies in each treated well. Experiments were carried out in triplicate.

2.9.7 TRANSWELL MIGRATION ASSAY

Insert, containing a filter 8 µm pores were used for the transwell migration assay. Insert were placed in 24-well plates containing 750 µl of RPMI with 10% FCS which was used as a chemoattractant. $2.5x10^4$ cells suspended in 250 µl of serum-free media were added to each insert and incubated for 6-9 h or overnight under standard conditions. Next day, the media was removed from both upper and lower compartment (from the insert and the well). Cells were then scraped from the top surface of the insert with a cotton pad. The insert was washed with PBS twice followed by fixation with 500 ml of 4% formaldehyde for 20 min at room temperature. After washing the 4% formaldehyde with PBS twice, the insert was dipped in 500 µl hematoxylin for 1 h at room temperature. The membrane was cut from the insert carefully and placed it into a slide. Finally, 5 µl of DAPI was added on the coverslip, which was then replaced on the slide. Cells were then counted under the 40x microscope. Each experiment was performed in triplicate.

2.9.8 MATRIGEL INVASION ASSAY

Invasion assays were performed using Corning® Matrigel® Basement Membrane Matrix (10 ml). On day one, 24-well plates were coated with diluted Matrigel (5 mg/ml to mg/ml) in a serum-free cold RPMI. Next, 100 µl of the diluted matrigel was added into the upper chamber of 24-well transwell and incubate at 37°C overnight. After 24 h incubation of the Matrigel, 750 µl RPMI media, supplemented with 10% FBS, was added to the lower chamber as a chemo-attractant. $2.5x10^5$ cells suspended in 200 µl of serum-free media were added to the upper chamber. Cells were then treated with siRNA for 48 h incubation at 37°C, cells that had invaded through the pores onto the lower surface of the filters were fixed with 100% methanol for 30 s. Next, the membrane was washed with

distilled H_2O whereas the non-invaded cells at the top of the Matrigel membrane were removed. Cells present on the bottom of the membrane represented the invading cells. The membranes were stained with 1% crystal violate (Sigma-Aldrich) and the total number of invading cells was counted under an Olympus CX41 microscope at 40x magnification. Each experiment was repeated in triplicate.

2.10 FLOW CYTOMETRY

2.10.1 CELL-CYCLE DISTRIBUTION AND SUB-G_1 REGION ANALYSIS

The cell cycle analysis was performed in LNCaP, 22RV1, DU145 and PC3 by flow cytometry. The cells (1.2×10^4 cells/well) were seeded in 6-well plates and were treated with 25, 10, 30 and 25 μM of HU210 or DMSO respectively for 24 h. Then, cells were harvested by trypsinization and centrifugation. The cell pellets were resuspended and fixed in 70% ice-cold ethanol/PBS, pelleted and resuspended in buffer containing 50 μg/ml RNase A and 50 μg/ml propidium iodide (PI). Then, cells were incubated in the dark for 15 min on ice and then analysed by FACScan benchtop cytometer. The cell cycle phases, sub-G1 (apoptosis), G1, S, and G2/M, were measured by fluorescence emission at 617 nm (FL-2) after treating with RNase A and staining with PI. Each experiment was repeated in triplicate.

Flow cytometry acquisition was performed on an FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, CA, USA) with CellQuest Pro software version 4.0.2 (Becton Dickinson Bioscience, CA, USA). A gate was drawn around the viable cells. Ten thousand gated events were analyzed per sample. The data obtained were further analyzed with FlowJo 9.1 software (Tree Star, ORE, USA) by plotting selected cells into a histogram displaying PI intensity which correlates with DNA content calculated by using Watson Pragmatic integration.

2.11 GENOMIC ALTERATIONS ANALYSIS

2.11.1 TRADITIONAL SANGER SEQUENCING-DIRECT SEQUENCING

The sequence of the human *CNR1* gene (88879767-88848583) was retrieved from the public databases by BLAST search ((http://www.ensembl.org/Homo_sapiens/Gene/Sequence?g=ENSG00000118432;r=6:88849583-88876078). The genomic DNA sequence was found in http://www.ensembl.org, enabling us to construct the primers covering 1Kb upstream from exon 1 (upstream promoter), 1Kb upstream from exon 3 (second promoter) and the coding region of the human *CNR1* gene with the expected size ~590 - 600 bp for each PCR products (Figure 2.9). Four prostate cancer cell lines (22RV1, LNCaP, DU145 and PC3) as well as one immortalized epithelial prostate cell line (PNT1a), were screened for alteration in *CNR1* by direct sequencing the coding region and the two promoter regions.

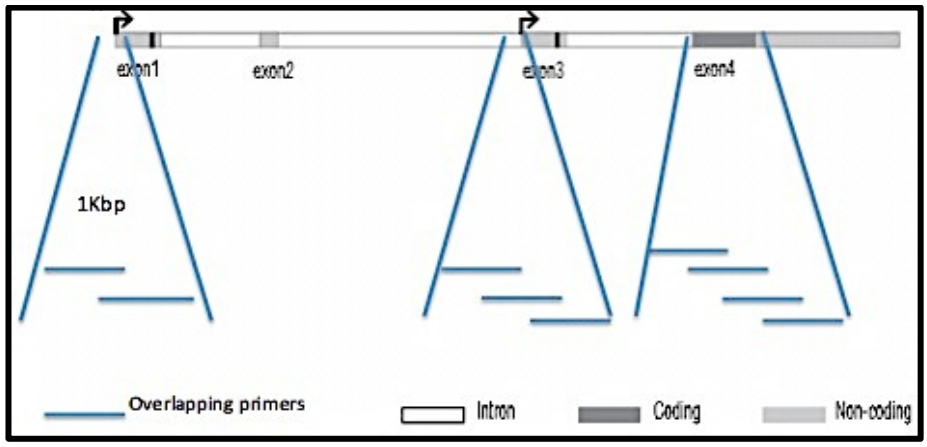


Figure 2.9 Schematic representations of genomic DNA of the gene *CNR1*, and the position of the primers. Arrows indicate the position of the promoters.

All primers applied were ordered from Sigma. PCR reactions with genomic DNA template were performed as described in section 2.2.1.4. Primer sequences, PCR thermos-cycle program are shown in (Table 2.9) and (Table 2.10). Direct DNA sequencing was performed in Genome Centre (QMUL).

Table 2.9 The primers for Strong, weak promoter and coding regions for *CNR1* sequencing

No.	PCR Primers	Sequence	Conditions annealing Tm	Expected size	Genome position
1	CNR1p1.1R CNR1p1.1F	5' CGTAGCCCCAAACTTTGCT 3' 5' CCTACGGAGCCCAACTGTTT 3'	61°C	637bp	2928-3565
2	CNR1p1.1F CNR1p1.1R	5' GGAGGAGGAGGAAGAGGAGA 3 5' GCGGAAAAGAAGTGGAGAAG 3'	61°C	612bp	3453-4065
3	CNR1p2.1F CNR1p2.1R	5' ACGGAGTCTCGCTCTGTCG 3' 5 GCCTGACACAAAAGTAAGTCTTCA3'	64°C	639bp	17838-18477
4	CNR1p2.1F CNR1p2.1R	5' AAGGCAATGAGCATTCTTGAG 3' 5'GGTGCTATTATCCCCATTTTCA 3'	64°C	601bp	18325-18926
5	CNR1p2.1F CNR1p2.1R	5' GGGTGCAGTGCTTGCTCTA 3' 5' ACCAGCCTCCTACTGATGGA 3'	64°C	630bp	18834-19464
6	CNR1e4.1F CNR1e4.1R	5' TCCAAGAGTAGGGGTCATGTG 3' 5' TCATTTGAGCCCACGTACAG 3'	64°C	595bp	24259-24854
7	CNR1e4.2F CNR1e4.2R	5' GTCGATCCTAGATGGCCTTG 3' 5' CCCACCCAGTTTGAACAGAA 3'	64°C	579bp	24780-25359
8	CNR1e4.3F CNR1e4.3R	5' GCCTTCCTACCACTTCATCG 3' 5' CCAGCAGATGATCAACACCA 3'	64°C	618bp	25224-25842
9	CNR1e4.4F CNR1e4.4R	5' GGATGGGAAGGTACAGGTGA 3'	64°C	641bp	25743-2684

5' CCCTGGAGAATGGAGTTTGA 3'

Table 2.10 PCR program for *CNR1* gene

No.	Step	Temperature (C°)	Time
1	Initial denaturation	95°C	5mins
2	Denaturation	95°C	30sec
3	Annealing	61°C strong promoter 64°C coding region 64°C weak promoter	45sec
4	Elongation	72°C	1min
5	Go to 2	-	35 cycles
6	Final elongation	72°C	10mins
7	Stop	4°C	Forever

2.11.2 DNA MICROSATELLITE ANALYSIS

Specific primers were designed for microsatellite analysis. The forward PCR primer was labeled with fluorescent dye (FAM) (Table 2.11). Genomic DNA was PCR-amplified. For this, 100 ng/µl of genomic DNA was mixed with 1 µl of each primer; 10 mM of 1µl dNTP, 5µl PCR buffer containing 15 mM MgCl2, 0.4 µl of Taq polymerase and distilled water in total volume of 50 µl. PCR thermos-cycle started with a denaturation step of 3 min at 95ºC and continued with 35 cycles, containing; a 30 sec denaturation segment at 95ºC, a 45 sec annealing segment at 61ºC, and

a 30 sec elongation segment at 72ºC. The final elongation step was extended to 10 min in order to allow all the products to be fully extended.

Two different prostate samples groups have been analysed using microsatellite analysis. 13 UK prostate cancer samples were analysed for polymorphism at the *CNR1* promoter region in the Genome Centre (QMUL). The other group is from China (Genomic DNA from 96 blood samples taken from Chinese non-cancer prostate patients and the other group from 104 gDNA blood samples taken from Chinese prostate cancer patients). The samples were analysed in China for polymorphisms in the *CNR1* promoter region.

Table 2.11 primers for Strong promoter region for microsatellite instability analysis

PCR Primer	Sequence	Expected Size
CNR1p1-Forward	5' FAM–CCACGGGAGCGGCCTTGC-	150 bp
CNR1p1-Reverse	5' –GCGCATCGCCAACACCTTCC-	

2.11.2 NEXT-GENERATION SEUENCING-NGS

The genomic regions containing all exons of *CNR1* and upstream promoter region were amplified using Fluidigm Access Array using primers designed by the company (Fluidigm, Cambridge, UK). PCR product of each primer pair was assessed by Agilent Bioanalyser and further processed for next generation sequencing on an Illumina MiSeq system (Illumina, San Diego, CA). Sequencing data were aligned with Bowtie 2 (Langmead and Salzberg 2012) and variants were called with VarScan 2 (Koboldt, Zhang et al. 2012) using Genome Reference Consortium human build 37 (GRCh37). A mutation was recorded only when the mutant allele frequency is 10 or more.

3 CHAPTER THREE RESULTS CHARACTERISATION OF *CNR1* STATUS IN PROSTATE CANCER

3.1 GENETIC CHANGES OF *CNR1*

Molecular genetic analysis by our team and others revealed a frequent deletion in the 6q15 region in prostate cancer and the downregulation of a number of genes at this region (Verhagen, Hermans et al. 2002, Liu, Chang et al. 2007, Shan, Ambroisine et al. 2010, Boyd, Mao et al. 2012). Our SNP array data showed that 6q15 was deleted in 53% of prostate cancer samples. In addition, 6q15 was deleted in up to 46% of prostate cancer samples, as detected by FISH analysis (Shan 2010), further supporting that the deletions in 6q15 are frequent events and that *CNR1* is a candidate TSG at this region in prostate cancer.

3.1.1 LACK OF MUTATIONS OF CNR1 GENE IN PROSTATE CANCER

To investigate the potential of *CNR1* inactivation through mutation in prostate cancer, and to characterise *CNR1* status in the prostate cancer cell lines for further functional studies, I sequenced the *CNR1* gene in the prostate cancer cell lines, using the traditional Sanger method with overlapping PCR primers as described in (Section 2.8). I determined the *CNR*1 sequences in the human prostate cancer cell lines 22RV1, LNCaP, DU145, PC3 and VCaP, and one immortalized prostate epithelial cell line, PNT1a. The sequencing data of *CNR1* showed a clear indiviual sharp and evenly spaced peaks. Moreover, the lack of baseline noise in the sequences as shown in the DNA sequencing chromograph indicates good quality sequencing data with clean nucleotide peaks (Figure 3.1A).

My results showed no mutations in *CNR1* coding and promoter regions of the prostate cancer cell lines 22RV1, LNCaP, DU145 and VCaP and PC3.

However, a polymorphism of DNA sequence with or without a 6bp (CCTTCC) was found in the promoter of *CNR1* gene (Figure 3.1B). Most of the cancer cell lines have the six bases,compared to the common *CNR1* sequence and the PNT1a immortalised prostate epithelial cells without the six bases at the position 3250-3256 bp in the analysed prostate cancer cell lines (Figure 3.2).

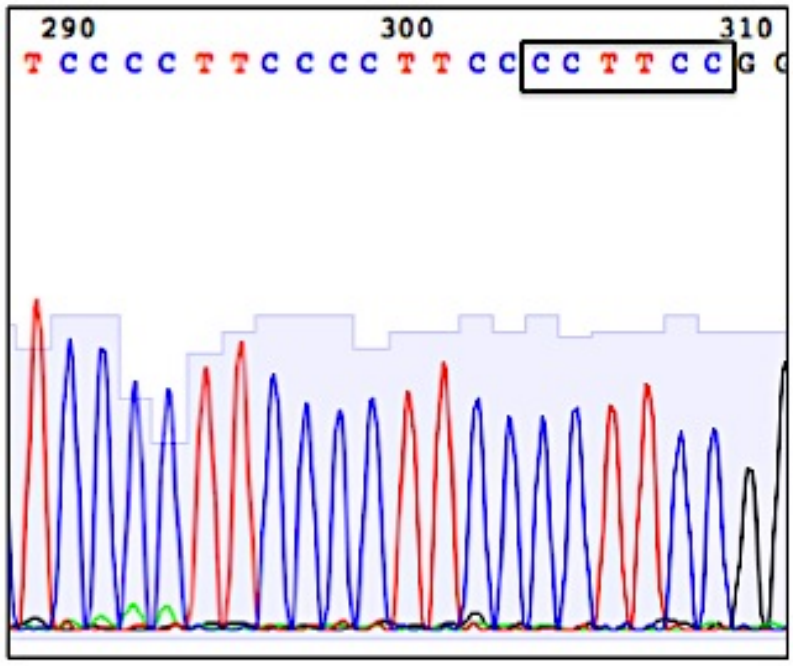


Figure 3.1A DNA sequencing chromatograph of *CNR1* from prostate cancer cell line 22RV1. The sequence lacks of baseline noise as shown in the DNA sequencing chromograph, indicating a clean nucleotide peak. No mutaion was found in *CNR1* coding and promoter regions.

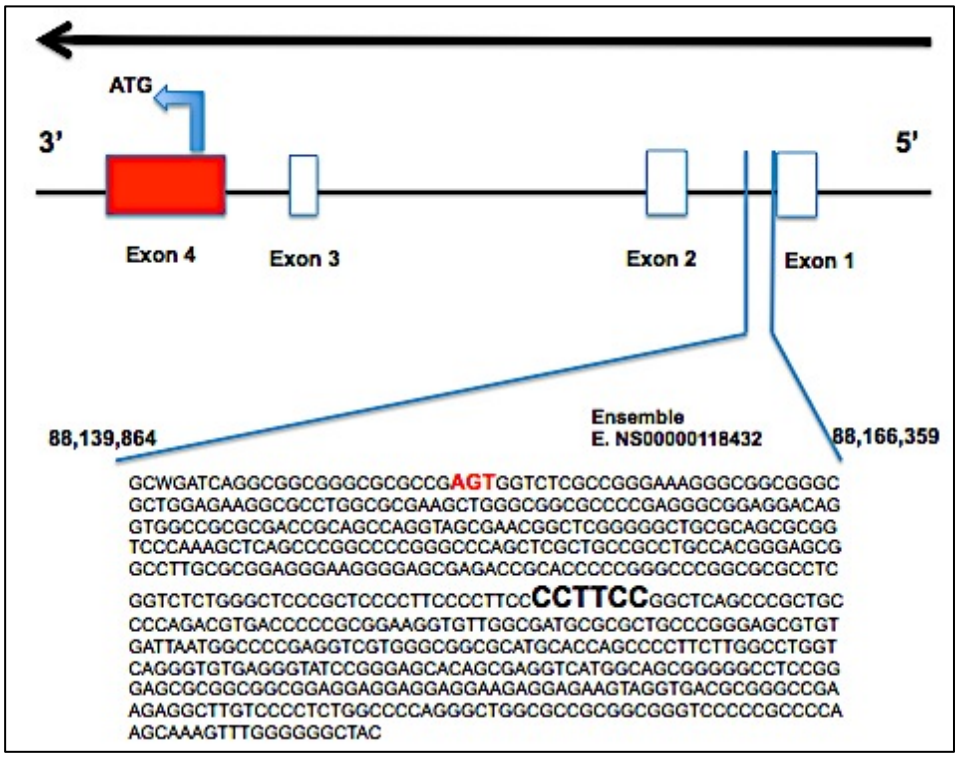


Figure 3.1B The genomic DNA sequence of CNR1 gene (http://www.ensembl.org), The construction of the primers covering 1Kb upstream from exon 1 (upstream promoter). The 6bp polymorphism sequence is present in human genome (black bold letters).

Figure 3.2 Multiple sequence alignment using ClustalW comparing the sequenced genomic DNA of prostate cancer cell lines with the *CNR1* database sequence in the upstream promoter region. The 6bp polymorphism sequence is present in human genome.

Gene mutation is another mechanism leading to the inactivation of TSGs. To further investigate the *CNR1* inactivation, a screen for mutations in *CNR1* was performed on human prostate cancer samples, using more advanced techniques than traditional Sanger sequencing, which can be a laborious task particularly when performed on a large genomic region with a large cohort of samples. Fluidigm genomic amplification technology was used therefore in a combination with the next generation sequencing (NGS). Prostate cancer clinical samples were analysed using this method aiming at detecting any mutations in a gene of interest. The experiment was designed by Dr Lara Boyd and Dr Yong-Jie Lu in our team and performed by Dr Lara Boyd and the Genome centre (QMUL). The data analysis was performed by Mr Jacek Marzec (Centre for Molecular Oncology). The upstream promoter and coding region of *CNR1* we amplified by Fluidigm Access Array in 73 prostate cancer samples, and five prostate cancer cell lines (PC3, 22RV1, DU145, VCaP and LNCaP) and two immortalized prostate epithelial cell lines PNT1a and PNT2-C2, and analysed using next-generation sequencing. >100X depth was generated for all *CNR1* exon regions and the promoter. The data demonstrate that a somatic mutation in CNR1 is a rare event in prostate cancer.

Taken together, these results demonstrate that there are no mutations in *CNR1* coding and promoter regions. Polymorphism of 6bp sequence in the promoter region will be further investigated in the following section.

3.1.2 THE POLYMORPHISM IN CNR1 PROMOTER REGIONS IN PROSTATE CANCER- MICROSATELITE ANALYSIS

In the previous section 3.1, a polymorphism of 6bp was found in the promoter of *CNR1* gene by analysing the prostate cancer cell lines 22RV1, LNCaP, DU145 and VCaP and PC3. *In silico* analysis, using *Ensemble*, I identified this polymorphism (variant rs147446147) to be located within the upstream promoter region of the *CNR1* gene (Figure 3.1B).

All the cell lines only contain the longer allele type (154bp), the recently reported variant rs147446147 (with 6bp polymorphism), except in PC3 cells where both the shorter (147bp) and longer (154bp) alleles are present as compare to PNT1a (Figure 3.2).

Then I analysed genomic DNA of 13 blood samples taken from UK prostate cancer patients for the polymorphism. We found six out of 13 blood samples contain the longer allele sequence 154 bp (homozygous) in the promoter region of *CNR1* gene (Figure 3.3).

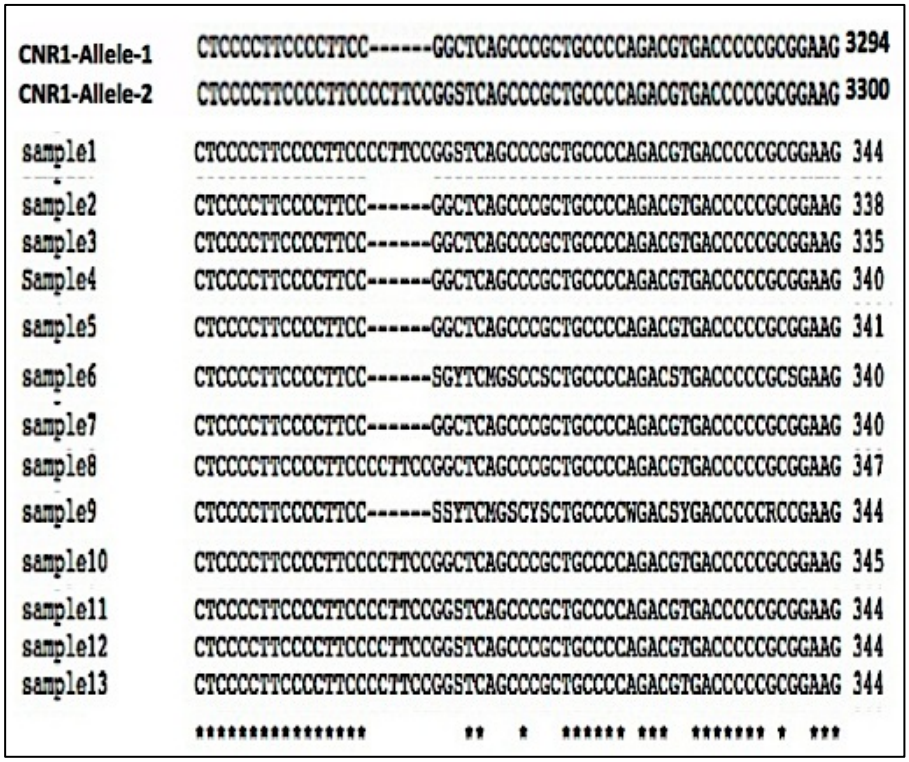


Figure 3.3 Multiple sequence alignment using ClustalW comparing the sequenced genomic DNA of blood sample taken from prostate cancer patients with the CNR1 database sequence in the upstream promoter region. The 6bp polymorphism sequence is present in human genome with Homo-A2 (homozygous allele-2) 6bp longer and Hetero-A1 (heterozygous allele-1) 6bp shorter.

I further investigated the presence of the 6bp polymorphism and its potential effect on transcription factor binding sites, in case it may influence *CNR1* gene expression by altering transcription factor binding to the promoter

region. A TF regulates transcription of its target gene by specifically binding to the transcription factor-binding site (TFBS) in the gene promoter region. I found potential TFBs for five TFs; TFII-1, STAT4, c-Ets-1, Elk-1 and GR-alpha overlap with the polymorphism 6bp sequence (Table 3.1). This overlapping causes the addition of another TFBS for each TF (Figure 3.4). As illustrated in Figure 3.4, the sequence in the promoter region with and without the 6bp polymorphism. It appears that the insertion results in additional TFBS for each TF. Also detected changes of the TFBS position of TFII-I and GR-alpha after the insertion of 6bp polymorphism sequences.

Table 3.1 Potentially predicted transcription factor binding sites at RS147446147 **region using Promo (version 8.3 of transfac).**

Start position	End position	Sequence Similarity	Potential TFBSs
290	294	CCTTC	GR-alpha
296	300	CCTTC	
302	306	CCTTC	
290	295	CCTTCC	TFII-I
296	301	CCTTCC	
302	307	CCTTCC	
290	295	CCTTCC	STAT4
296	301	CCTTCC	
302	307	CCTTCC	
291	297	CTTCCCC	c-Ets-1
297	303	CTTCCCC	
303	309	CTTCCGG	
291	299	CTTCCCCTT	Elk-1
297	305	CTTCCCCTT	
303	311	CTTCCGGCT	

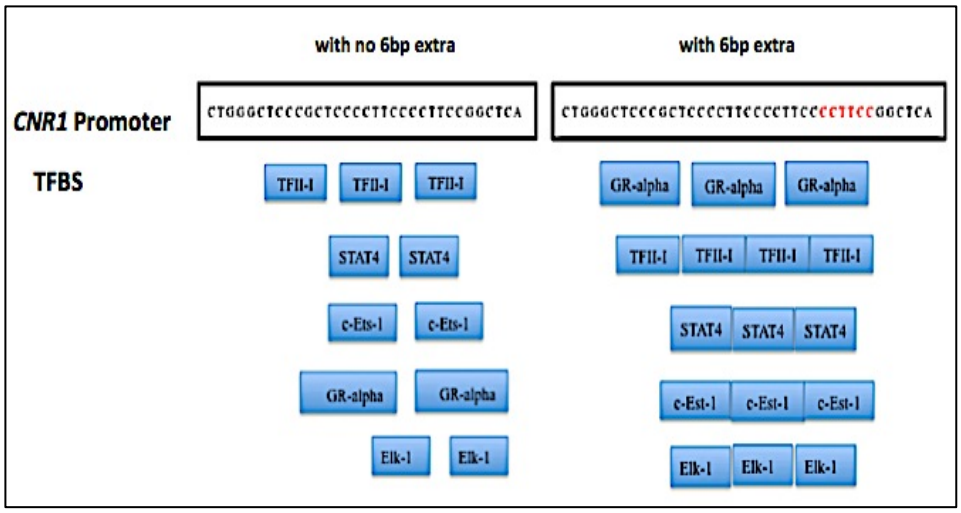


Figure 3.4 Potential TFBS based on human *CNR1* upstream sequence are marked by blue boxes: TFII-1, STAT4, c-Ets, GR-alpha and Elk-1. It appears that for each TFBS that I identified, there are two TFBS located in the upstream promoter regions of *CNR1*. However, with the 6bp polymorphism sequence, it causes the addition of another TFBS. The alignment shows the position of the TFBS in different positions of the promoter regions.

Consequently, I further investigated if there is a difference of the frequency of these polymorphism alleles between prostate cancer and non-cancer control groups in the Chinese population, where blood samples were available. 96 Chinese non-cancer and 97 Chinese cancer blood samples were subjected to microsatellite analysis using sequencing gel and we found that the longer allele sequence was around 154 bp (homozygous), which correlates to the polymorphism with the 6bp is more common in cancer samples compared to non-cancer samples (Table 3.2). In Chinese cancer samples, 67 (69.79%) samples with longer allele sequence while in the non-cancer samples, 58 (60.42%) with longer allele sequence. These results suggest that the longer allele sequence is more common than the shorter one in the Chinese population.

Table 3.2 Microsatellite analysis results of rs147446147 for Chinese non-cancer and cancer prostate blood samples

Sample	Homozygous (147) no insertion	Homozygous (154) with insertion	Heterozygous (147/154)	Total
Non-cancer China	4 (4.1%)	58 (60.42 %)	34 (35.42 %)	96
Cancer China	4 (4.1%)	67 (69.79 %)	26 (27.08 %)	97

I further investigated whether there is any association between allelic-specific and prostate cancer risk. A Fisher exact test was carried out to test the allele-specific association with prostate cancer risk (Table 3.3). Using the two-tailed test, the P-value equals 0.307 and consequently the association between cancer and the homozygous longer allele is not statistically significant. Our finding showed that the longer allele (154 bp) is not associated with prostate cancer risk in Chinese population. Therefore, this CNR1 promoter polymorphism is not involved in prostate cancer development at least in the Chinese population.

Table 3.3 Statistical analysis of difference in rs147446147 alleles between Chinese non-cancer and cancer samples using microsatellite analysis.

Samples	147bp allele	154 bp allele	*P* Value*
Non-cancer	42	150	
Cancer	34	160	0.307
Total	76	310	

*A Fisher exact test-two-tailed.

3.2 GENE EXPRESSION OF CNR1 IN PROSTATE CANCER

I determined the expression of *CNR1* transcripts by qRT-PCR for the prostate cell lines used in this study. I observed that the cell line DU145 presented the highest level of *CNR1* transcript expression. Slightly higher expression levels were also observed in 22RV1 and LNCaP cells compared to the PNT1a and PNT2 immortalised prostate epithelial cells. PC3 and VCaP cells had very low levels *CNR1* expression (Figure 3.5).

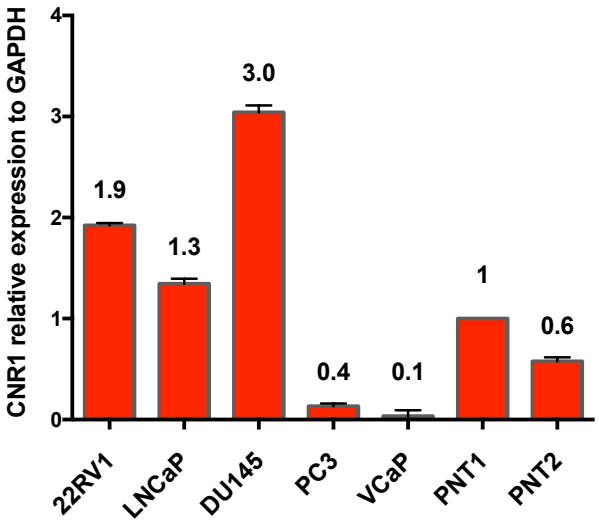


Figure 3.5 *CNR1* mRNA expression in prostate cancer cell lines. *CNR1* mRNA expression in all prostate cancer cell lines and two immortalized epithelial cells lines as assessed by RT-qPCR analysis. Expression of *CNR1* measured by RT-qPCR relative to the expression of the housekeeping gene *GAPDH*. Endogenous *CNR1* levels were normalised with respect to the *CNR1* level in PNT1a. Values are determined from two independent experiment performed in triplicate.

3.3 EXPRESSION OF CNR1 RECEPTOR IN PROSTATE CANCER CELLS AT PROTEIN LEVEL

3.3.1 ANTIBODY SELECTION

Having determined *CNR1* expression in 22RV1, LNCaP and DU145, PC3 and immortalised epithelial cells at mRNA level (section 3.2.1), I aimed to determine protein expression by western blot analysis of whole cell lysates from the prostate cancer cell lines. The specificity of an antibody is crucial for examining protein expression in cells. Therefore the antibodies against CNR1 products were carefully selected and antibody-working conditions were optimised prior to use in further studies. First I evaluated a number of CNR1 antibodies to identify a specific antibody, as a recent study reported a series of commercial CNR1 antibodies which were non-specific for western blot and IHC (Grimsey, Goodfellow et al. 2008). I therefore reviewed the literature and tested all the CNR1 antibodies available at the time, including the following commercially available CNR1 antibodies: C-terminal antibodies from Abcam (ab23703) and Merck (209550), one N-terminal antibodies produced by Santa Cruz as well as one antiserum (L13) developed by Prof. Elphick (QMUL), by western blotting using human brain tissue, where CNR1 is highly expressed (Egertova and Elphick 2000), as a positive control.

Cell extracts from the prostate cancer cell lines 22RV1, LNCaP, DU145, PC3, VCaP, the two immortalized prostate epithelial cell lines PNT1a and PNT2, and a human brain lysate as a positive control, were blotted for CNR1. Western blot analysis with the antibody from Abcam (ab23703) exhibited multiple bands including two prominent bands ~63 kDa and ~49 kDa in most cell types, considered to reflect the presence of two different states of the protein which may correspond to CNR1 and CNR1b protein respectively (Egertova and Elphick 2000) (Figure 3.6). The Abcam antibody revealed an immunoreactive band for most prostate cancer cell lines at 49 kDa which was slightly larger in size compared to the brain lysate (<49kDa) (shown in Figure 3.6). The immunoreactive band at 63

kDa was detected in most cell types including the positive control (brain lysate). In addition, there are strong bands with molecular weights at ~35 kDa that cannot be ignored since the positive control also showed a strong and specific band at 35 kDa.

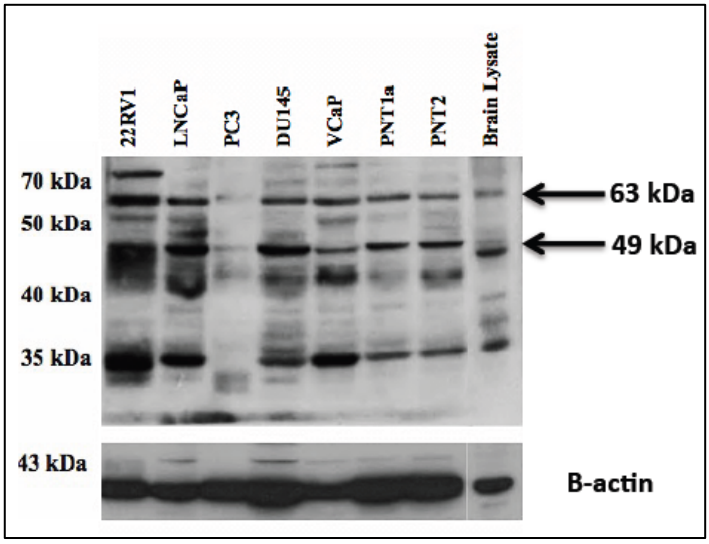


Figure 3.6 Western blot of CNR1 for human CNR1 receptor proteins expressed in prostate cancer cell lines. Western blot of proteins extracted from prostate cancer cell lines using Abcam (ab23703) antibody directed against CNR1. β-actin was used as loading control. 40 µg protein was loaded per lane and blotted for CNR1. One blot representative of three independent experiments.

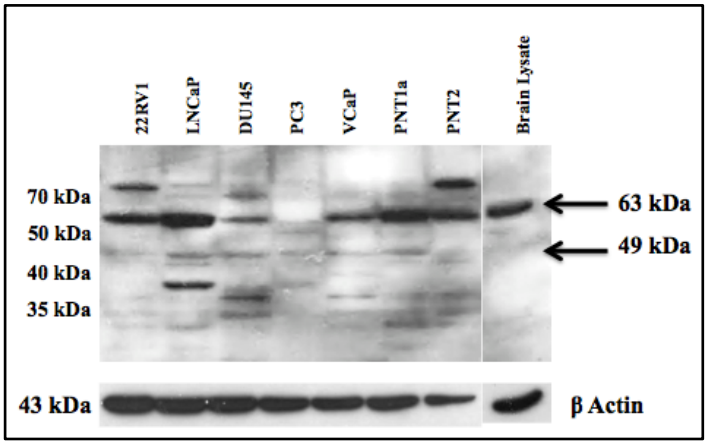


Figure 3.7 Western blot of CNR1 for human CNR1 receptor

proteins expressed in prostate cancer cell lines. cell lysates were prepared for separation on polyacrylamide gels. 40µg protein was loaded per lane and blotted for CNR1, using (L13) Antibody directed against CNR1. β-actin was used as loading control. One blot representative of three independent experiments.

Antibody L13 (Prof Elphick, QMUL) also showed a CNR1 specific banding pattern at ~63 kDa and ~49 kDa (Figure 3.7). Multiple bands were obtained using CNR1 antibody from Merck (209550). Optimization of the Merck (209550) antibody did not improve detection of the predicted CNR1 protein (Figure 3.8). Also, multiple bands were obtained between the molecular weights ~45 kDa and ~35 kDa with the Santa Cruz (sc-10066) antibody, but little at 63 and 49 kDa (Figure 3.9).

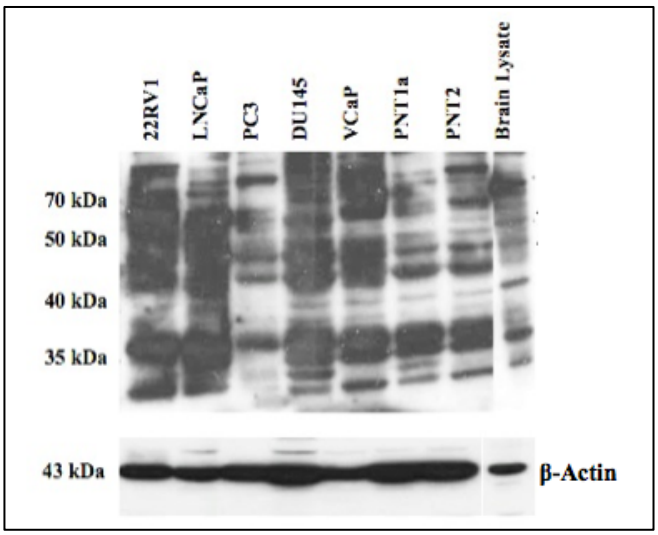


Figure 3.8 Western blot of CNR1 for human CNR1 receptor proteins expressed in prostate cancer cell lines. Cell lysates were prepared for separation on polyacrylamide gels. 40µg protein was loaded per lane and blotted for CNR1 using Merck (209550) antibody directed against CNR1. β-actin was used as loading control. One blot representative of three independent experiments.

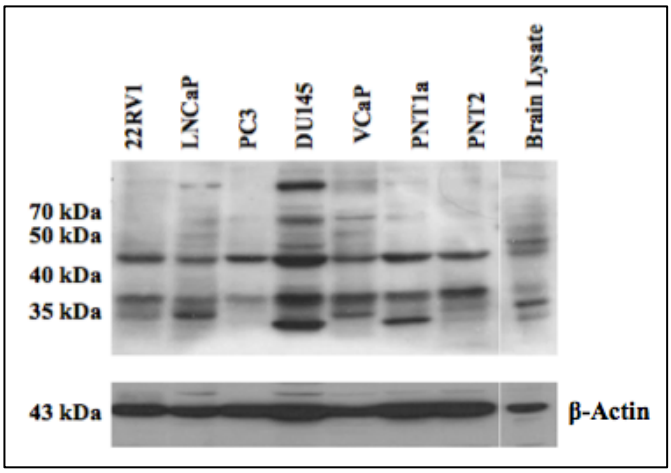


Figure 3.9 Western blot of CNR1 for human CNR1 receptor proteins expressed in prostate cancer cell lines. cell lysates were prepared for separation on polyacrylamide gels. 40µg protein was loaded per lane and blotted for CNR1 using Santa Cruz antibody directed against CNR1. β-actin was used as loading control. One blot representative of three independent experiments.

The results demonstrated the lack of CNR1 specificity of Merck (209550) and Santa Cruz (sc-10066) antibodies. However, Abcam (ab23703), and L13 antibodies had the potential to detect the endogenously expressed CNR1 receptors in prostate cancer cell lines. Therefore, the Abcam (ab23703), and L13 antibodies were employed for further optimisation.

3.3.2 INHIBITION OF CNR1 GLYCOSYLATION PROTEIN IN LNCAP CELLS

Both antibodies ab23703 and L13 (Prof. Elphick, QMUL) detected CNR1 signals by western blot analysis corresponding to ~63 kDa and 49 kDa (Figure 6 & 7). It has been reported that N-glycosylation contributes to the molecular weight of the protein species and inhibition of glycosylation can shift the bands detected by western blot (Andersson, D'Antona et al. 2003). To further evaluate which of the two antibodies are better for CNR1 detection, LNCaP cells were treated in the absence or presence of N-glycosylation inhibitor tunicamycin for 72h. Tunicamycin inhibits N-

glycosylation by blocking the transfer of N-acetylglucosamine-1-phosphate (GlcNAc-1-P) from UDP-GlcNAc to dolichol-P, thereby decreasing the formation of dolichol-PP-GlcNAc and inhibits glycosylation. Western blot analysis with Abcam anti-CNR1 and L13 antibody was performed on whole cell lysates collected from untreated and tunicamycin-treated cells (Figure 3.10).

Using the Abcam antibody, treatment with (2 µg/ml) tunicamycin for 72 h, resulted in a decrease in the bands at ~63 kDa and 49 kDa with a molecular shift to about ~40 kDa in LNCaP cell extract. However, this ~40 kDa band was not detected by the L13 antibody. Instead, the L13 antibody detected a new band in addition to the 63 kDa and 49 kDa bands, at about ~90 kDa in LNCaP cell extract. Surprisingly, the 49 kDa band disappeared completely which indicated that 49 kDa band might represent another glycosylated CNR1 protein. These data showed the specificity of the Abcam antibody that was therefore employed in all further western blotting.

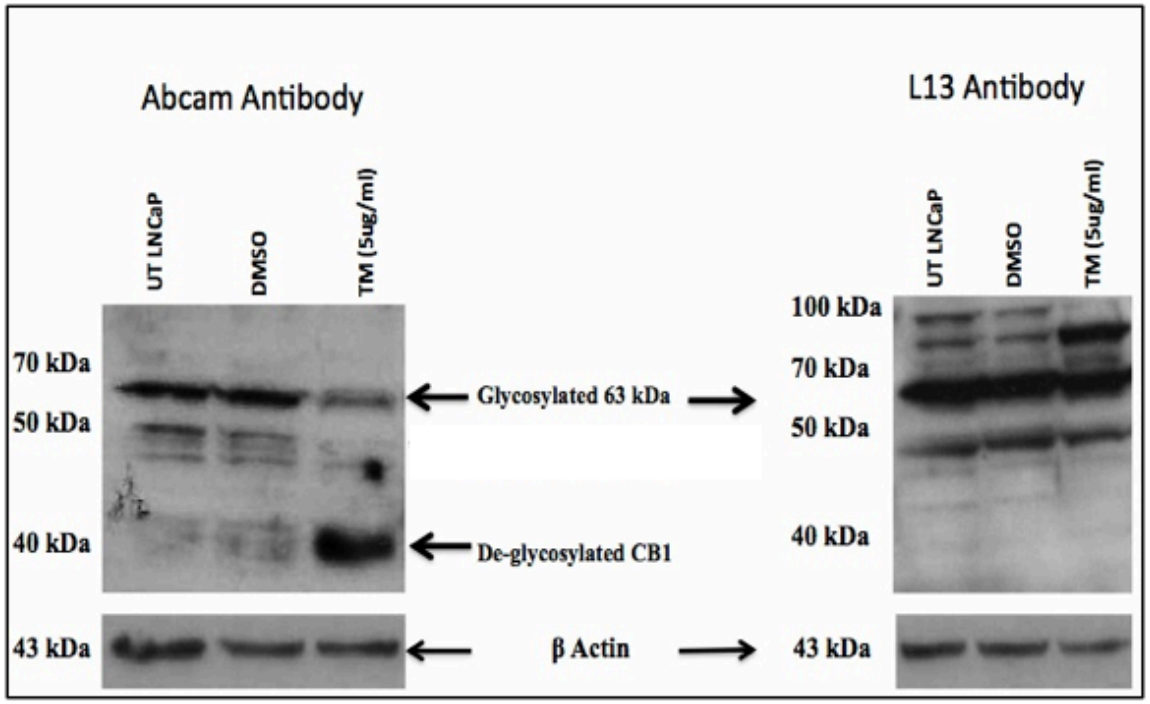


Figure 3.10 CNR1 is highly glycosylated in LNCaP cells. LNCaP cells were treated with tunicamycin for 72 h. Lysates were prepared from untreated LNCaP cells (UT), control cells treated with DMSO (-), and LNCaP cells treated with 2 μg/ml tunicamycin (TM) for 72h in 10% serum media at 37°C. Total cell lysates (50μg/lane) were then processed and immunoblotted with Abcam rabbit polyclonal antibody and L13 antibody to detect CNR1. The membranes were re-probed with ß-actin to control for loading. The arrows indicate the glycosylated and shifted form of CNR1. Similar findings were observed in two independent experiments.

3.3.3 *Determining CNR1 knockdown in LNCaP cells and further confirming the specificity of the antibody*

3.3.3.1 Knockdown of *CNR1* in LNCaP using double transfection of siRNA

In the previous section 3.3.2, I determined that the anti-CNR1 antibody from Abcam detects the expression of CNR1 and CNR1b. To further confirm the specificity of the Abcam antibody, I used gene silencing via siRNA. CNR1 was knocked down by a specific siRNA in the prostate cancer cell line LNCaP.

A double siRNA transfection method was performed in the LNCaP cells, where *CNR1* mRNA is expressed. After 48 h post transfection, using the double transfection protocol, a 90% reduction in *CNR1* mRNA was observed in cells transfected with the *CNR1*-targeting siRNA compared to non-targeting siRNA transfected cells (Figure 3.11).

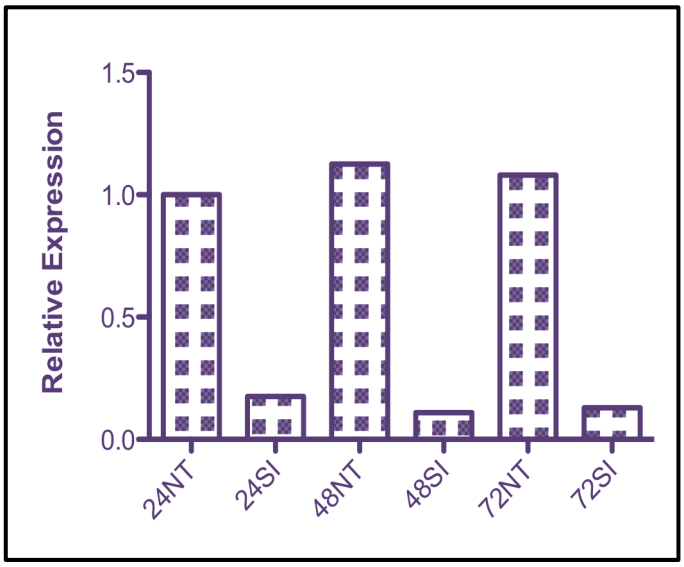


Figure 3.11 Relative expression of *CNR1* mRNA in LNCaP cells using double transfection method. Initial siRNA transfection of pre-seeded LNCaP cells, followed by re-transfection after 48h. Cells were transfected twice (Double transfection with 100nM siRNA complexes and Oligofectamine reagent). The expression level of

CNR1-targeting siRNA in LNCaP cells (Si) was measured by RT-qPCR relative to the expression of the housekeeping gene *GAPDH* and normalized to the non-targeting siRNA transfected LNCaP cells. mRNA expression represents one experiment.

The effect of knockdown of CNR1 on protein expression in LNCaP cells was determined using western blot analysis. A significant difference in the relative density of the two isoforms in cells double-treated with CNR1-targeting siRNA in comparison to non-targeting transfected cells was found (Figure 3.12). Therefore, these results demonstrate that the double-transfection method effectively knocked down both forms of CNR1 receptors in LNCaP cells and further confirmed the specificity of the Abcam antibody. Interestingly, other bands showed reduction after siRNA transfection as well. The possible explanation for this reduction in protein density is that it may be the product of CNR1 receptors that have been subjected to proteolysis or during protein extraction.

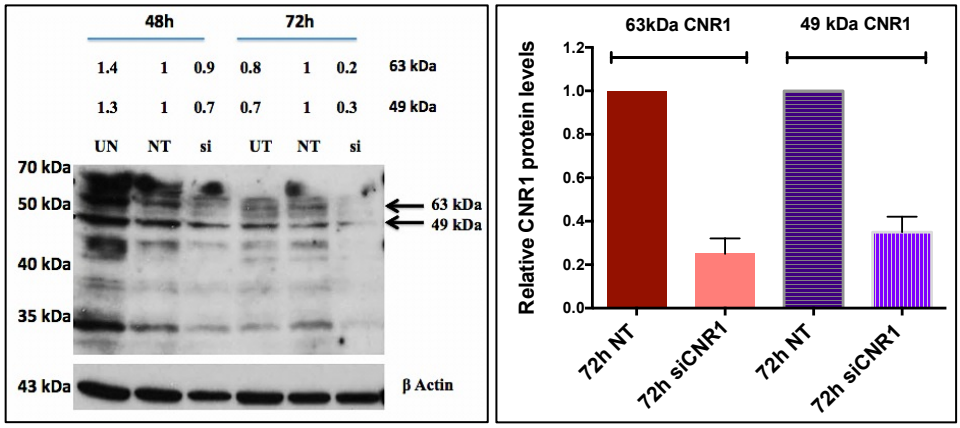


Figure 3.12 Knockdown of CNR1 in LNCaP cells using double transfection method. Using Abcam antibody 1:100 dilution, LNCaP cells were seeded in 6 well plates for 24 h prior to transfection. The cells were then transfected with oligofectamine and CNR1-targeting siRNA /non-targeting reagents. Cells were re-transfected with 100 nM siRNA complexes 48 h after the first transfection. Western blot analysis of CNR1 expression in untreated LNCaP cell (UT), LNCaP cells transfected with non-targeting siRNA (NT), LNCaP cells transfected with CNR1-targeting siRNA (si). Total cell lysates 40µg/lane). β-actin was used as a loading control with expected size at 43 kDa. The values above the figures

represent the relative density of the bands normalised to Actin. One blot representative of three independent experiments.

3.3.3.2 Knockdown of *CNR1* in LNCaP using reverse transfection of siRNA

To reduce the exposure time of cells to the transfection reagents, transfection of CNR1 siRNA was performed on LNCaP cells in suspension. After 24 h, 90% of the volume of the media containing transfection complexes was replaced with fresh media to reduce the concentration of complexes and minimize toxicity to cells. The relative expression of *CNR1* decreased with up to 80% after 24 h of the transfection in comparison to non-targeting siRNA transfected cells (Figure 3.13).

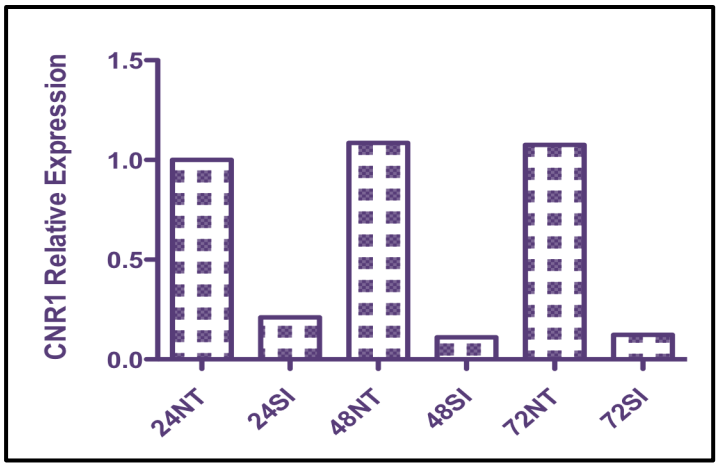


Figure 3.13 Relative expression of *CNR1* mRNA in LNCaP cells using reverse transfection method. The expression level of *CNR1*-targeting siRNA in LNCaP cells (si) was measured by qRT-PCR relative to the expression of the housekeeping gene *GAPDH* and normalized to the non-targeting siRNA transfected LNCaP cells (NT) after 24 h, 48 h, and 72 h post transfection. mRNA expression is representative of one experiment.

Protein expression of CNR1 in LNCaP cells transfected with CNR1-targeting siRNA and non-targeting was examined using western blot analysis 72 h post-siRNA transfection. Most of the CNR1 protein expression was knocked down in CNR1-targeting siRNA transfected LNCaP cells in comparison to non-targeting siRNA transfected cells (Figure 3.14). These results also demonstrate that the reverse transfection method effectively knocked down both forms of CNR1 receptors in LNCaP cells.

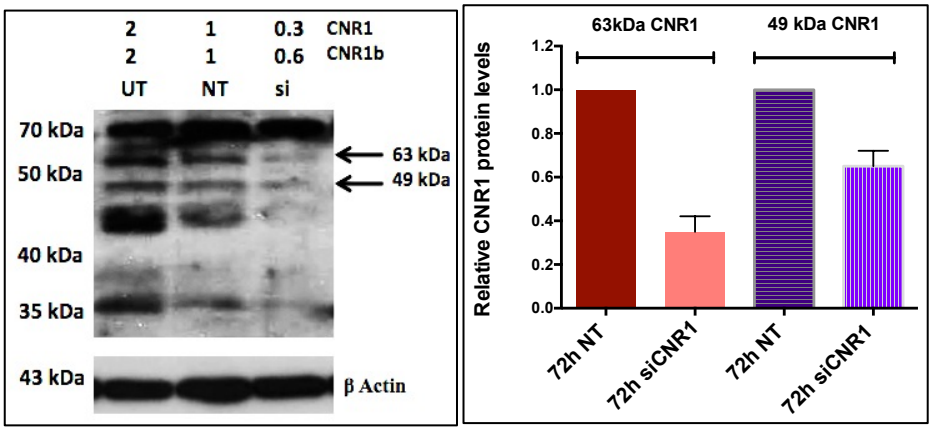


Figure 3.14 Knockdown of CNR1 in LNCaP cells using reverse transfection of siRNA after 72h post transfection. Using Abcam antibody (1:100 dilution, LNCaP cells, oligofectamine and siCNR1 reagent were incubated all at the same time. Western blot analysis of CNR1 expression in untreated LNCaP cells (UT), CNR1 expression in LNCaP cells transfected with non-targeting siRNA (NT), CNR1 expression in LNCaP cells transfected with CNR1-targeting siRNA (si). Total cell lysates 40µg/lane). β-actin was used as a loading control with expected size at 43 kDa. The values above the figures represent the relative density of the bands normalised to Actin. One blot representative of three independent experiments.

My data demonstrate that both transfection methods did knock down *CNR1* expression at both mRNA and protein levels and further confirmed the specificity of the Abcam antibody.

3.3.4 KNOCKDOWN OF *CNR1* IN 22RV1 CELLS TRANSFECTED WITH *CNR1* CONFIRMED THE SPECIFICITY OF THE ABCAM ANTIBODY

To further confirm the specificity of the antibody, I used 22RV1 cells that overexpressed *CNR1*, previously generated in our team (Shan 2010). The *CNR1* overexpressing cells were previously generated in our laboratory using a pcDNA3.1 (+) plasmid containing the *CNR1* coding region. Several clones had been selected by culturing cells in the presence of geneticin G418 (1mg/ml).

The expression levels of *CNR1* mRNA were examined using TaqMan qRT-PCR analysis to measure the increases in *CNR1* expression in 6 clones compared to the control 22Rv cells transfected with empty vector. It showed that *CNR1* mRNA levels were 120 and 113 fold higher in clone 1, and 5 respectively, in comparison to the control cells (Figure 3.15). The overexpression of *CNR1* in the stably transfected 22RV1 clone 1 was selected for continued examination of Abcam antibody by knocking down *CNR1* in these cells using the siRNA reagents.

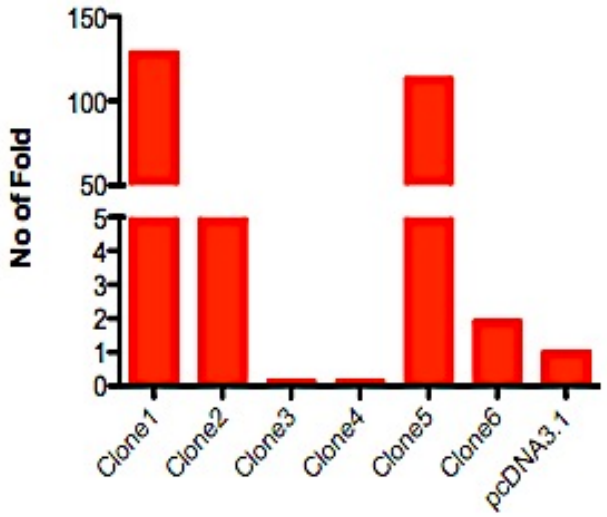


Figure 3.15 Overexpression *CNR1* at the mRNA level in stably transfected 22RV1 clones using TaqMan qRT-PCR analysis. The expression of *CNR1* RNA levels in *CNR1* stably transfected 22RV1 clones were measured by TaqMan RT-qPCR, relative to the expression

of the housekeeping gene *GAPDH* and normalized to the pcDNA3.1 (+) transfected 22RV1 cells as a control. mRNA expression is representative of one experiment.

3.3.4.1 Knockdown of *CNR1* in stably transfected 22RV1 clone 1 cells at mRNA levels

CNR1-targeting siRNA and non-targeting siRNA were transfected respectively into Clone 1 of the *CNR1* stably transfected 22RV1cells. The decreases in *CNR1* mRNA levels were measured by TaqMan-based gene expression detection (TaqMan probe: *CNR1* Hs00275634_m1) and the RT-qPCR reactions were performed using ABI 7900 Real-Time PCR system. The housekeeping gene (*GAPDH*) was used as an endogenous control. The expression level of *CNR1*-targeting siRNA in stably transfected 22RV1 cells (SI) was measured by RT-qPCR normalised to the expression of the housekeeping gene *GAPDH* and relatively to the non-targeting siRNA transfected 22RV1 (NT) cells after 24 h, 48h and 72h transfection. The results showed that the relative expression of *CNR1* gene was silenced by up to 75% at 24 h post-transfection in *CNR1*-targeting siRNA transfected 22RV1 cells in comparison to non-targeting siRNA transfected cells (Figure 3.16).

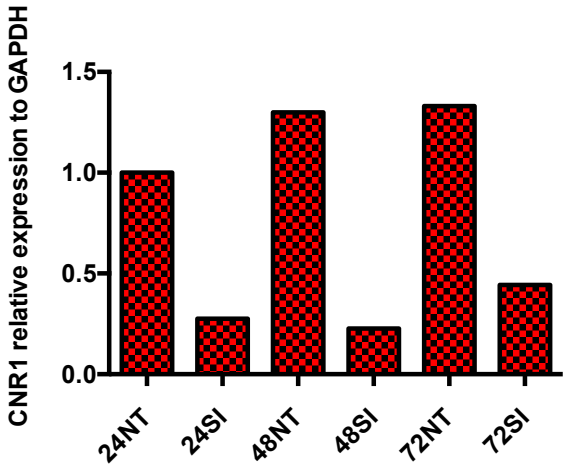


Figure 3.16 Relative expression of *CNR1* in CNR1-stably transfected 22RV1 cells clone 1 with and without *CNR1* knockdown. mRNA expression is representative of one experiment.

3.3.4.2 Knockdown of CNR1 in 22RV1 cells was determined by immunofluorescent imaging

To further confirm expression and knockdown of CNR1 at the protein level, the Abcam ab23703 antibody was used in the immunofluorescent imagining of 22RV1 cells clone 1 with and without CNR1 siRNA transfection.

The efficiency of the CNR1 knockdown at protein level was examined by immunofluorescence after 24 h siRNA transfection. In my study, the siCNR1 treatment substantially reduced the expression of CNR1 protein in 22RV1 cells as compared with non-targeting siRNA transfected cells (Figure 3.17). Importantly, the protein expression of CNR1 was found to be downregulated in these cells after 24 h of treatment. It was also clear that CNR1 was located to the cell membrane and the cytoplasm (green signal) in the 22RV1 cells clone 1 as expected (Figure 3.17). These results confirm that rabbit polyclonal anti-CNR1 Abcam antibody ab23703 is capable of detecting CNR1 protein in 22RV1 cells.

Overall, my results in this section showed that the expression of *CNR1* at the mRNA level correlated with the protein levels in prostate cancer cell lines. Furthermore, the Abcam antibody proved to be specific for CNR1 receptors and can be used for further functional studies to investigate the role of CNR1 in prostate cancer.

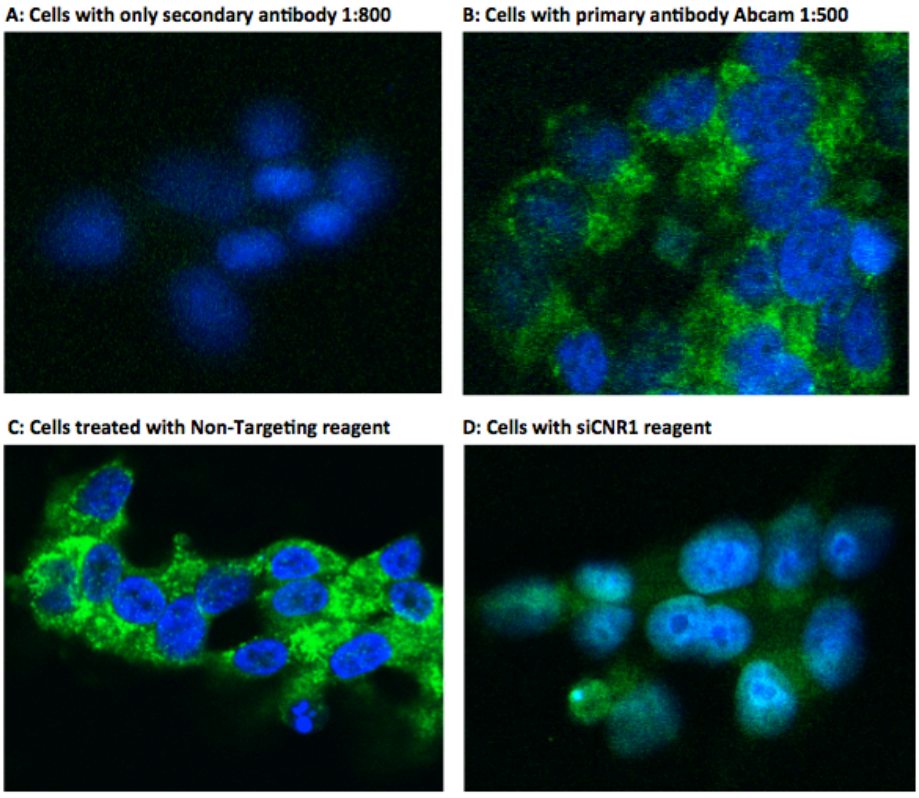


Figure 3.17 Detection of CNR1 receptors under expression using confocal fluorescence microscopy. Images showing immunofluorescent localisation of CNR1 immunoreactivity in stably transfected 22RV1 cells. Cells treated with (**A**) secondary antibody, (**B**) primary antibody, (**C**) Cells transfected with non-targeting reagent-siRNA, (**D**) Cells transfected with the siCNR1 reagent for 24 h and stained with antibody Alexa Fluor 488 conjugate. CNR1 receptors exhibit membrane and cytoplasmic distribution. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) blue and CNR1 receptors (green) at 1000x magnification. Similar findings were observed in two independent experiments.

3.4 DISCUSSION

3.4.1 CHARACTERISATION OF CNR1 STATUS IN PROSTATE CANCER

Molecular genetic analysis by our team and others revealed a frequent deletion in the 6q15 region in prostate cancer and the downregulation of a number of genes at this region, including *CNR1* (Verhagen, Hermans et al. 2002, Liu, Chang et al. 2007, Shan, Ambroisine et al. 2010, Boyd, Mao et al. 2012). I aimed to further investigate the potential of *CNR1* as a TSG in prostate cancer and characterise *CNR1* status in available prostate cancer cell lines for further functional studies.

Tumourigenesis frequently occur when DNA sequences of genes encoding proto-oncogenes or tumour suppressor genes (TSGs) are altered. Alterations in proto-oncogenes may affect their function, resulting in constitutive activation and promotion of cell division (Bishop 1987), while any defects including deletion or mutations in TSGs also contribute to cancer formation. First, I sequenced the coding region and 1kbp strong regions of the *CNR1* gene in five prostate cancer cell lines, 22RV1, LNCaP, DU145 and PC3 and a non-malignant immortalized epithelial prostate cell line, PNT1a. No mutations were detected in the coding or the promoter regions of *CNR1*. To further investigate any mutations in *CNR1* in prostate cancer patients, a large number of clinical samples was analysed using a Fluidigm genomic amplification technology in combination with the next generation sequencing. However, after comparing the sequences of the tumours with paired normal clinical samples, there was no evidence of somatic mutations in the *CNR1* gene. It has been reported that a high frequency of G→A mutation in the *CNR1* gene was detected in eosophageal cancer (Bedoya, Meneu et al. 2009). Of the 139 tissues from the 29 eosophageal cancer patients, homozygous G/G was found in 15 and heterozygous G/A were detected in 85 samples. The patients with an A/A type had a reduced survival time in comparison to a G/G type. In addition, a study in colorectal cancer has shown that patients, who had genotype G/A or A/A, had a shorter overall survival time than G/G wild- type patients (Bedoya, Rubio et al. 2009). These studies

suggest that this point mutation inactivated *CNR1* in cancers (Bedoya, Rubio et al. 2009, Meneu-Diaz, Bedoya et al. 2011). In prostate cancer, somatic mutations in the *CNR1* gene appear not to be a main mechanism of inactivation.

There is an increasing focus on the role of polymorphisms in prostate cancer development and progression. In my study, I detected the 6bp polymorphisms in *CNR1* promoter region. Transcription factors (TFs) control the transcription of its target gene by binding to the transcription factor-binding sites (TFB) in the promoter region (Spitz and Furlong 2012). Identifying polymorphisms in the promoter would be a strong indication of disruption of transcription if they overlap with transcription factor binding sites. Using transcription factor binding site prediction tools (TRANSFEC), I found that the 6bp polymorphism causes the addition of the following transcription factors: c-Ets-1, Elk-1, GR-alpha, STAT4, and TFII-1. The promoter region controls and regulates mRNA transcription, making it the most important regulatory region. In my study, I found that the addition of 6pb polymorphisms generally correlated with *CNR1* expression in prostate cancer cell lines. All the prostate cancer cell lines only contain the longer allele type (154bp), except PC3 cells where both the shorter (147bp) and longer (154bp) alleles are present. Interestingly, PC3 cells have much lower *CNR1* expression compared to the other cancer cell lines. However, the effects of the polymorphism in the *CNR1* promoter would have to be confirmed, for example by using a luciferase promoter-reporter assay in prostate cancer cells. Due to time limitations, these studies were not performed during my PhD thesis.

As there are very limited number of cell lines for the correlation of the polymorphism and *CNR1* expression, I further investigated the 6bp polymorphisms in the *CNR1* gene promoter for its involvement for prostate tumourigenesis in Chinese cases and controls, for which we have access to samples. The frequency of the longer alleles was slightly more common in Chinese normal than cancer samples. However, statistically, the longer allele (with insertion) was not associated with prostate cancer risk.

In conclusion, polymorphism tests for *CNR1* gene (with long sequences) were not associated with prostate cancer incidence of the Chinese population, which discourages the further investigation of its role in UK samples, which we have not collected.

3.4.2 *EXPRESSION OF CNR1 GENE IN PROSTATE CANCER*

It is known that *CNR1* is mainly distributed in the nervous system, however, this receptor has also been detected in various non-neuronal tissues including prostate (Miller and Devi 2011). I found *CNR1* gene expression in both several human prostate tissues and cell lines, regardless of whether the samples were benign or malignant.

A recent report demonstrated that *CNR1* expression was silenced in human colorectal cancer due to methylation of the *CNR1* promoter and that loss of *CNR1* accelerated intestinal tumour growth in vivo (Wang, Wang et al. 2008). According to the findings in our group (Shan 2010), *CNR1* is downregulated in prostate cancer cell lines and clinical samples compared to normal BPH samples. Therefore, I further investigated the expression of *CNR1* in prostate cancer.

According to my results, gene expression of *CNR1* in LNCaP and 22RV1, DU145 but not PC3, are higher than immortalized epithelial prostate cells PNT1a and PNT2 at mRNA level. This finding is in slight contrast to the previous findings for the *CNR1* by our group (Shan 2010) where under-expression of *CNR1* in prostate cancer cell lines LNCaP, 22RV1, DU145 compared with BPH. The reason for the different findings is unclear, but maybe due the use of different control samples and also quantitative RNA detection method including Taqman probe/primers. Shan et al 2010 performed RT-qPCR using probe/primers detecting the entire exon 4, which lied within a single exon which could be the reason for the low expression of *CNR1* reported in prostate cancer cell lines (Shan 2010). I

used probe/primers span exon-exon junctions that bind the coding region of *CNR1* to ensure that the primers cannot associate with gDNA.

The discrepancy in results between these two methods may lie in the specificity of the primers. Another reason for this conflict in our results may also be due to using different controls that do express the *CNR1* gene. In my RT-qPCR assay, I included two non-malignant control cells PNT1a and PNT2 to support the expression of *CNR1* in prostate cancer cell lines, whereas in previous RT-qPCR study, BPH was used as a positive control. It was reported that *CNR1* expression was found to be high in BPH, prostate cancer clinical samples and PrEC cells (ORELLANA-SERRADELL, POBLETE et al. 2015). However, Other published papers showed that *CNR1* was expressed in most prostate cancer cell lines including 22RV1, LNCaP, DU145 which supports my findings (Ruiz-Llorente, Sanchez et al. 2003, Sanchez, Ruiz-Llorente et al. 2003, Sarfaraz, Afaq et al. 2006, Chung, Hammarsten et al. 2009).

In addition I found that PC3 has low expression of *CNR1* at mRNA and protein level, which is consistent with our previous findings (Shan 2010) and other published studies, where low expression of *CNR1* at mRNA (Kamiyama, Fukasawa et al. 2013) and protein level (Christopher John Fowler 2009) were also detected at low level in PC3 cells. Moreover, this low expression was supported by the deletion of the *CNR1* genomic region, which results in the low expression of *CNR1*.

CNR1 protein expression assessment with a specific anti-CNR1 antibody would greatly improve the understanding of expression and the role of CNR1. However, recent studies have reported that a series of commercial CNR1 antibodies do not specifically detect CNR1 by western blot or IHC analysis (Grimsey, Goodfellow et al. 2008). Therefore the antibodies against *CNR1* products were carefully tested and antibody-working conditions were optimised prior to use in further studies. First I evaluated a number of CNR1 antibodies in including C-terminal and N-terminal antibodies in prostate cancer cell lines as well as human brain tissues and found Abcam antibody works well but not the other antibodies reviewed in

the literature. I found that antibodies corresponding to C-terminal amino acids of the human CNR1 are more likely to detect CNR1 receptors in prostate cancer cell lines and brain tissues. However, Rabbit polyclonal Merck (209550) antibody, a recombinant protein consisting of the first 77 amino acid of rat CNR1 receptor, failed to detect CNR1 receptors in prostate cancer cell lines and brain tissues.

The anti-CNR1 antibodies ab23703 and L13 detected endogenously expressed CNR1 by western blot analysis giving bands at ~63 kDa (CNR1) and ~49 kDa (CNR1b) in the prostate cancer cell lines (LNCaP, 22RV1, DU145, VCaP) which are consistent with previous published publications (Shire, Carillon et al. 1995, Ruiz, Miguel et al. 1999, Sarfaraz, Afaq et al. 2005, Czifra, Varga et al. 2009, Brown, Cascio et al. 2010). However, western blotting with both ab23703 and L13 antibodies are also associated with additional bands at other molecular weights. A possible explanation is that these immunoreactive protein bands are in fact artefact of the isolation process.

My western blot study supported my evidence for the poor specificity of the N-terminal antibodies for CNR1. One of the particular concerns was the detection of multiple proteins with different sizes to the CNR1 isoforms and failed to detect the positive control. A possible explanation for the inefficiency of this antibody is that the protein conformation might change during the sample denaturation affecting the antibody recognition of the epitope by the antibody (Bass, Wilkinson et al. 2016). Also, although specific bands at ~45 kDa and ~35 kDa were identified by sc-10066, these molecular weights differ to the size commonly reported for this antibody at ~49 to ~63 kDa.

Previous studies have shown that CNR1 can be glycosylated at one or two sites in the N-terminal tail of the receptor generating a protein with a molecular mass of 63 kDa and 49 kDa (Song and Howlett 1995, Egertova and Elphick 2000, Porcella, Marchese et al. 2002). Alternative spliced isoforms have been reported in rat and human (Shire, Carillon et al. 1995) A possible explanation for the 49 kDa band is that this protein is an alternative spliced isoform of the glycosylated CNR1 receptor. I evaluated the Abcam and L13 antibodies for their ability to detect glycosylated CNR1

proteins and whether inhibition of glycosylation affects the size of CNR1 protein detected by western blot using ab23703 and L13 antibodies. I found that the Inhibition of glycosylation by tunicamycin treatment in LNCaP cells induced the emergence of a prominent band at ~40 kDa when ab23703 antibody was used. Therefore, the 63 kDa and 49 kDa bands correspond in mass to the glycosylated CNR1 proteins, which were supported by previous studies (Shire, Carillon et al. 1995, Song and Howlett 1995, Egertova and Elphick 2000). In contrast, the use of L13 antibody using the same approach did not show the low molecular weight band after treating LNCaP cells with tunicamycin and there was no apparent change of the density of ~63 kDa band. While it is difficult to explain the western blot results using L13, this experiment led to the selection of ab23703 antibodies for further application.

Further validation of antibodies was carried out by targeting specific genes in mammalian cells for knockdown analysis, siRNA techniques have been widely adopted for functional gene studies (Elbashir, Harborth et al. 2001). The introduction of siRNA into target cells is critical for gene silencing. Thus, I further confirmed the specificity of the anti-CNR1 antibody ab23703 as I knocked-down *CNR1* using double and reverse transfection of cells with siRNA. The idea of using two different approaches was to insure that both CNR1 and CNR1b were knocked down at the protein level. I found that both siRNA transfection protocols were efficient in decreasing CNR1 at protein levels in LNCaP cells using western blot analysis. My data demonstrate that the reverse transfection method is more effective than the standard siRNA gene knockdown and improved the knockdown of CNR1 expression at protein levels.

Finally, to continue the story of confirming the specificity of the ab23703 antibody, I used immunofluorescence (IF) microscopy, as it is also a broadly applicable method used to assess the expression levels of CNR1 receptors (Pisanti, Picardi et al. 2011). The specificity of the antibody was confirmed. CNR1 is a cannabinoid receptor localised at the plasma membrane. Stably transfected 22RV1 cells, was used as a positive control, as it was demonstrated to have high *CNR1* mRNA expression using RT-

qPCR analysis. In my studies, a decrease in CNR1 protein expression was observed in the stably transfected 22RV1 cells following siCNR1 treatment. Importantly, the protein expression of CNR1 was found to be under-expressed in these 22Rv1 siCNR1 targeted cells after 24 h of treatment. The results were in agreement with previous IHC analysis that showed reduced receptor levels in the cell membrane (Shan 2010).

My findings are also consistent with several other published studies showing that *CNR1* was expressed in prostate cancer cell lines, including 22RV1, LNCaP and DU145, and PC3 but at different levels using immunofluorescence imaging analysis (Sarfaraz, Afaq et al. 2005, Chung, Hammarsten et al. 2009, Czifra, Varga et al. 2009, Brown, Cascio et al. 2010). The Abcam antibody applied here for IF analysis has been demonstrated to have high specificity in prostate tissue (Chung, Hammarsten et al. 2009). Chung et al had examined CNR1 expression using IHC analysis in 372 prostate cancer cases and 349 BPH samples (Chung, Hammarsten et al. 2009) and this study used the same CNR1 antibody (Abcam).

Overall, my results in this chapter confirmed the high expression of CNR1 in prostate cancer cell lines with low expression in PC3 at mRNA and protein levels. Also, it showed that the expression of *CNR1* at the mRNA level correlated with the protein levels in prostate cancer cell lines. The low expression of CNR1 at mRNA and protein level correlated with the presence of hetero-alleles in PC3 cells. Furthermore, the ab23703 antibody proved to be specific for CNR1 receptors and can be used for further functional studies to investigate the role of CNR1 in prostate cancer. Although it is possible that the differences generated between the studies were caused by different cell linages or cell culture conditions, such as different media and growth factors, the misrecognition of a nonspecific band at the expected size of the CNR1 protein might have been the main reasons for these conflicting results. The conflict between a potential tumour suppressor role and reported high expression of *CNR1* may by due to the difficulty in accurate detection of CNR1 protein expression caused by the uncertainty of a specific and reliable antibody or/and a possible reason for the discrepancy between gene and protein

expression is that other post-transcription and/or post-translation mechanisms affect CNR1 expression. Moreover, as *CNR1* is located at 6q15, one of the most frequently deleted regions in prostate cancer; DNA mutation or methylation may reduce the receptor activity. Also, deregulation in downstream signalling pathways in some of the patient samples may have caused the reduction of receptor activity.

Different observation supported the upregulation of CNR1 in prostatic adenocarcinoma tissues, and several cell lines including PC-3, DU-145, LNCaP, CWR22Rv1 and CA-HPV-10, as compared with normal prostate epithelial cells (Sarfaraz, Afaq et al. 2005, Chung, Hammarsten et al. 2009, Czifra, Varga et al. 2009, Brown, Cascio et al. 2010, Sharma, Hudson et al. 2014). These observations agreed with my findings where CNR1 was upregulated in prostate cancer cell lines including DU145, LNCaP, and 22RV1. Furthermore, an extensive study of human prostate cancer samples revealed the expression level of CNR1 was considerably higher in prostate cancer tissues than in normal prostate tissues (Chung, Hammarsten et al. 2009). Other cancer types including hepatocellular carcinoma (Xu, Liu et al. 2006) and pancreatic ductal adenocarcinoma showed an increased level of CNR1 expression in human pancreatic tumour cell lines as well as in biopsies of human pancreatic tumours, whereas in samples obtained from normal pancreatic tissue, mRNA levels for these receptors were very low or could not be detected (Carracedo, Gironella et al. 2006).

Relatively large amount of data have accumulated during the last decade about the role of CNR1 receptors in tumour generation and progression. In many cases, these reports showed that levels of CNR1 are increased in cancers including prostate cancer, a situation that frequently correlates with tumour aggressiveness (Malfitano, Ciaglia et al. 2011). It was found that patients with a tumour with higher CNR1 expression had a significantly higher proportion of Gleason scores 8–10, and metastases at diagnosis (Chung, Hammarsten et al. 2009), indicating that a high tumour CNR1 score is associated with prostate cancer severity of the disease and poor prognosis (Chung, Hammarsten et al. 2009). In other types of cancer,

Fowler et al, reported that the level of CNR1 receptor expression in colorectal cancer is associated with the tumour grade in a manner dependent upon the degree of CpG hypermethylation. They found that the high CNR1 is indicative of a poorer prognosis in stage II microsatellite stable tumour patients (Gustafsson, Palmqvist et al. 2011). Furthermore, CNR1 receptor levels are also increased and correlate with disease severity in human epithelial ovarian tumours (Messalli, Grauso et al. 2014) and have been proposed to be a factor of bad prognosis following surgery in stage VI colorectal cancer (Jung, Kang et al. 2013, Velasco, Hernández-Tiedra et al. 2015).

4 CHAPTER FOUR RESULTS
STUDY OF THE ROLE OF *CNR1* IN PROSTATE CANCER

Recent studies have demonstrated that *CNR1* regulates cell growth (Olea-Herrero, Vara et al. 2009, Preet, Qamri et al. 2011), cell proliferation (Sarfaraz, Afaq et al. 2006, Sarfaraz, Adhami et al. 2008, Xian, Park et al. 2010) and cell survival (Velasco, Hernández-Tiedra et al. 2015) (Pisanti, Picardi et al. 2011) in response to different stresses. The altered *CNR1* expression has been linked with the development and growth of various cancers (Bedoya, Meneu et al. 2009, Bedoya, Rubio et al. 2009, Larrinaga, Begoaa et al. 2010, Meneu-Diaz, Bedoya et al. 2011).

It is well known that CNR1 signalling pathways control cell proliferation, differentiation and survival. Deregulated CNR1 signalling pathways results in proliferation and contribute to the formation and progression of human cancers, including breast, pancreatic, lung and prostate cancers (Mimeault, Pommery et al. 2003, Sarfaraz, Afaq et al. 2005, Carracedo, Gironella et al. 2006, Xu, Liu et al. 2006, Ramer and Hinz 2008, Chung, Hammarsten et al. 2009, Zogopoulos 2015). Recently, the functional roles of CNR1 signalling in prostate cancer have been widely studied. Accumulated evidence suggest an active role for CNR1 involvement in the development and progression of prostate cancer (Pacher, BÅTkai et al. 2006). However, the exact role of CNR1 in prostate cancer is still unclear. In this study, I investigated a potential role of CNR1 in the development and progression of prostate cancer.

4.1 EFFECT OF CNR1 KNOCKDOWN IN PROSTATE CANCER CELLS

4.1.1 KNOCKDOWN OF CNR1 IN DU145 CELLS

I previously examined the expression of *CNR1* using qRT-PCR and western blotting, for mRNA and protein determination, respectively, in the prostate cancer cell lines 22RV1, LNCaP and DU145. However, the knockdown of CNR1 at the protein level in 22RV1 cells was difficult to detect using western blotting, therefore, I used DU145 cells as I found that *CNR1* was expressed in DU145 cells both at mRNA and protein levels. *CNR1* was knocked down in DU145 cells using the reverse transfection method as it was generally more effective than the standard transfection. To evaluate the functional significance of *CNR1* in prostate cancer, I silenced *CNR1* expression using siRNA in DU145 cells and then examined the expression of *CNR1* in DU145 cell line by RT-PCR and western blot analysis. As shown in the Figure 4.1A,B & C, RT-PCR and western blot analysis showed that the mRNA and protein expression levels of CNR1 were significantly decreased ($P<0.001$) by CNR1 siRNA with 80% reduction when compared with their expression in non-targeting siRNA. This demonstrates that *CNR1* could be knocked-down in the prostate cancer cell line DU145, which supported further functional analysis of the gene in these cells.

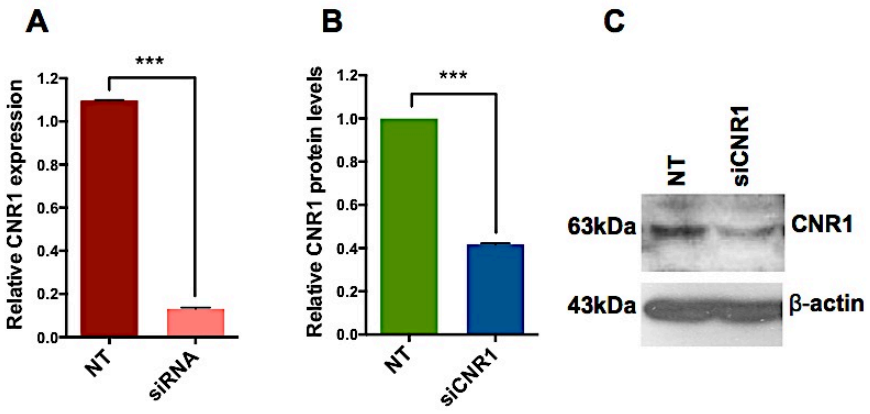


Figure 4.1 Knockdown efficiency of CNR1 in DU145 cells. (A) Relative mRNA levels of *CNR1* in DU145 cells quantified by qRT-PCR, showing a significant reduction after 48 hours of CNR1-siRNA (*siCNR1*) treatment compared to non-targeting siRNA (NT). *CNR1* mRNA levels were quantified relative to the housekeeping gene *GAPDH*. **(B)** Bar chart representation of CNR1 protein expression in DU145 cells, showing a significant reduction of CNR1 protein levels after 48 hours of *siCNR1* treatment compared to Non-Targeting siRNA (NT). Protein levels of CNR1 were quantified relative to the housekeeping protein β-actin. **(C)** Western blotting showing CNR1 expression levels after NT and *siCNR1* treatment. (A-B) Mean ± SEM of triplicate determinations (***P<0.001; by two-tailed t-test). C) One Western blot image representative of three independent experiments.

4.1.2 KNOCKDOWN OF CNR1 PROMOTES CELL PROLIFERATION IN THE DU145 PROSTATE CANCER CELLS

4.1.2.1 Cell Viability analysis

To evaluate the effect of CNR1 on cell proliferation, I analysed cell viability by the MTS cell proliferation assay. The results demonstrate a significant increase in DU145 cells proliferation/viability after down-regulation of *CNR1* (P<0.01) within 72h of siRNA transfection (Figure 4.2). This suggests that *CNR1* knockdown caused a significant increase in prostate cancer cell viability.

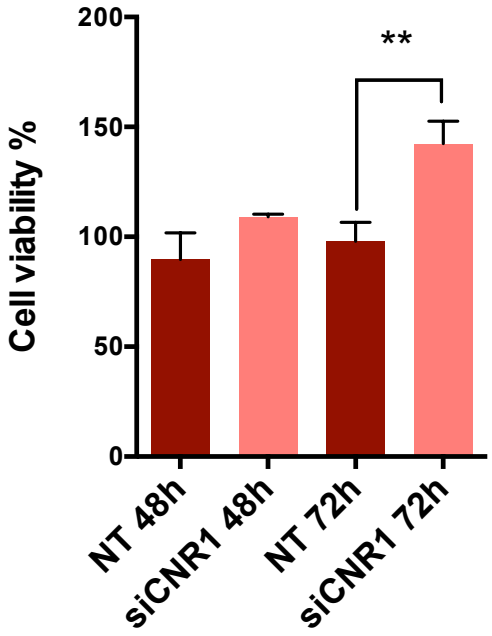


Figure 4.2 Cell viability upon CNR1 knockdown in DU145 cells. Cell viability levels of DU145 cells after non-targeting *siRNA* (NT) and *CNR1 siRNA* (*siCNR1*) for 48 and 72 hours treatment. Data are presented as means ± standard deviations of 3 independent experiments SEM± (**P<0.01; by two-tailed t-test).

4.1.2.2 Colony formation analysis

Moreover, to confirm the inhibitory effect of CNR1 on cell growth of prostate cancer cells, I used a colony formation assay after transfecting DU145 cells with siCNR1 for 48 h (Figure 4.3A). The number of the colonies was observed in both *CNR1* siRNA infected cells and the non-targeting siRNA cells. Clone formation rate of *CNR1* siRNA transfected cells (362.5 ± 17.5, n=2) in DU145 cells was significantly higher (P<0.05) compared to non-targeting siRNA cells (247.5 ± 17.50; n=2) (Figure 4.3B). The colonies in siRNA CNR1 knockdown cells were apparently bigger than non-targeting siRNA cells (Figure 4.3), but this was not measured. These data strongly support that with the decrease of CNR1, both the numbers and the sizes of the colonies are increased. Therefore, cell viability and colony formation assays indicate that *CNR1* may act as a potential tumour growth suppressor gene in prostate cancer.

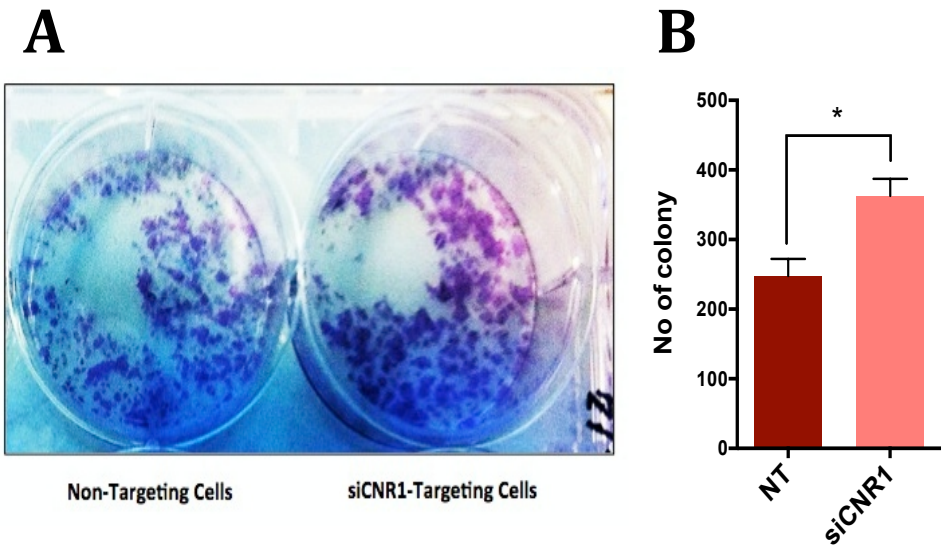


Figure 4.3 Colony formation assay in DU145 cell after CNR1 knockdown. (A) Image of colony formation assay of non-targeting siRNA (NT) and *siCNR1* DU145 cells. The cells were treated with siCNR1 for 48 h, then reseeded the cells for colony formation assay and were incubated for 7 days. Colony formation assay showing increased colony formation in numbers and sizes of colonies compared to non-targeting cells (NT). **(B)** Bar-chart of a number of colonies in non-targeting (NT) and siCNR1-targeting in DU145 cells treatment. Data are presented as means of 2 independent experiments ± SEM (*P<0.05; by two-tailed t-test).

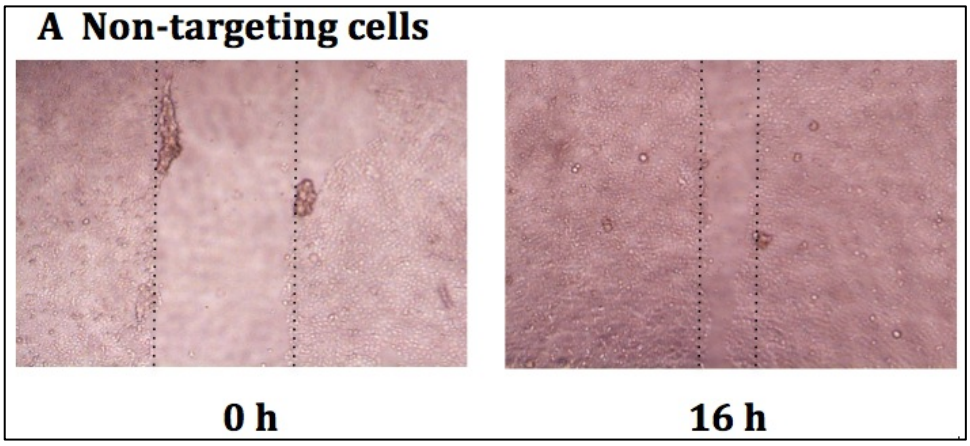
4.1.3 Effects of CNR1 silencing on migration and invasion of prostate cancer cells

The ability of tumour cells to migrate from the site of the primary tumour to invade surrounding tissues is a prerequisite for metastasis, which is the major cause of cancer-related mortality. Hence to evaluate the role of *CNR1* in prostate cancer, the effect of *CNR1* knockdown on cell migration and invasion were investigated.

4.1.3.1 Cell Migration

A scratch-wound assay was employed to study the effect of siCNR1 on DU145 cells. The scratch wound was induced by micropipette tips, and damaged cells were removed by changing of the culture media. As shown in Figure 4.4A and 4.4B, cells along the wound lines were viable after the scratch. The siCNR1 treated cells migrated rapidly to fill up the gap created by wounding and the gap was closed within 16h. The cells treated with non-targeting cells siRNA migrated much slower than siCNR1 treated cells and closed the gap after 16h incubation. These results implicate the involvement of *CNR1* in inhibiting cell migration.

To further confirm the role of *CNR1* in the progression of prostate cancer, Using the Transwell cell migration assay (Figure 4.5A), cells were seeded into the upper chamber with 1% fetal bovine serum (FBS) to promote migration to the lower chamber with 10% FBS levels. The number of migrating cells for non-targeting and CNR1 siRNA treated cells were 387.5 ± 26.5 and 719.5 ± 32.5 (averages ± SEM; n=2), respectively (Figure 4.5). Compared with non-targeting control, the migrated number of cells in *CNR1* siRNA treated cells significantly increased ($P<0.02$). The results suggest that silencing of CNR1 promoted the migration of prostate cancr cells, indicating that *CNR1* may act as tumour suppressor gene in prostate cancer.



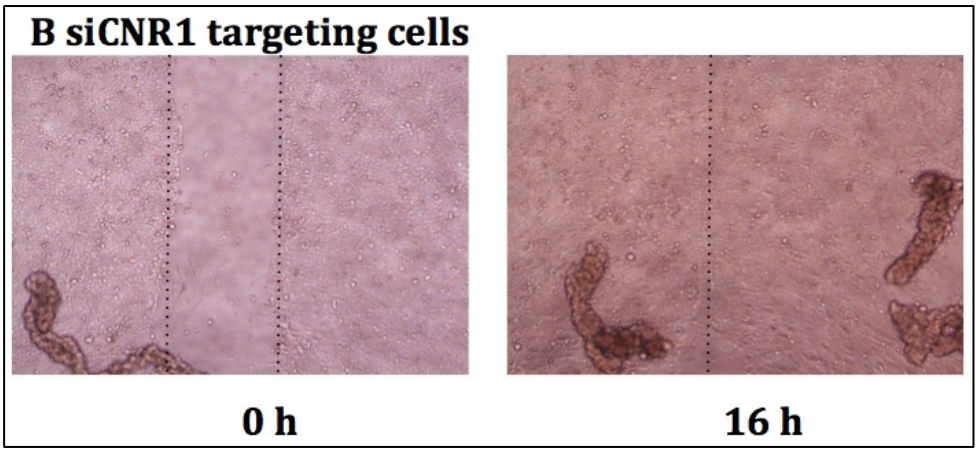


Figure 4.4 Scratch-wound assay in DU145 cells after CNR1 knockdown. (**A**). Non-Targeting siRNA in DU145 cells. (**B).** siCNR1 targeting cells. Cells were treated with siRNA-CNR1 (48h) migrated faster compared to Non-Targeting cells. Cells migration was evaluated 16 h after performing the scratch with 200 µl tips. Data are presented as one out of 3 independent experiments.

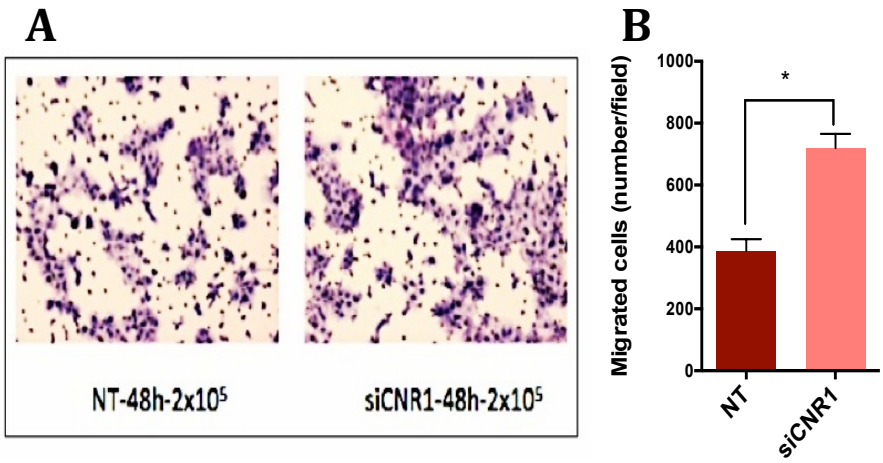


Figure 4.5 Transwell cell migration assay in DU145 cells after CNR1 knockdown. (A) Image of transwell cell migration assay of non-targeting siRNA (NT) and *siCNR1* treated DU145. **(B)** Bar-chart of a number of migrated DU145 cells upon NT and *siCNR1* treatment. Data are presented as means ± SEM of 2 independent experiments (*P<0.02; by t-test).

4.1.3.2 Cell invasion

To further demonstrate the role of CNR1 in the invasion of prostate cancer cells, I employed the loss of function approach to knockdown CNR1 expression in DU145 cells. I performed a Matrigel-coated Transwell invasion assay. The invasion was determined as cells penetrating through the Matrigel-coated Transwell chambers and travel through 8 **µ**m pores to the other side of the inserts (Figure 4.6A). The number of invading cells in non-targeting and CNR1 siRNA treated cells were 44.5 ± 1.5 and 77 ± 4 (averages ± SEM; n=2) respectively (Figure 4.6B). Compared with non-targeting control treated cells, the invading number in CNR1 siRNA treated cells significantly increased (P<0.05; Figure 4.6B); confirmed by crystal violate staining of the cells in the upper chamber of the transwell inserts. The results suggest that silencing of CNR1, promotes the invasion of prostate cancer cells, indicating that CNR1 plays an important role in invasive prostate cancer.

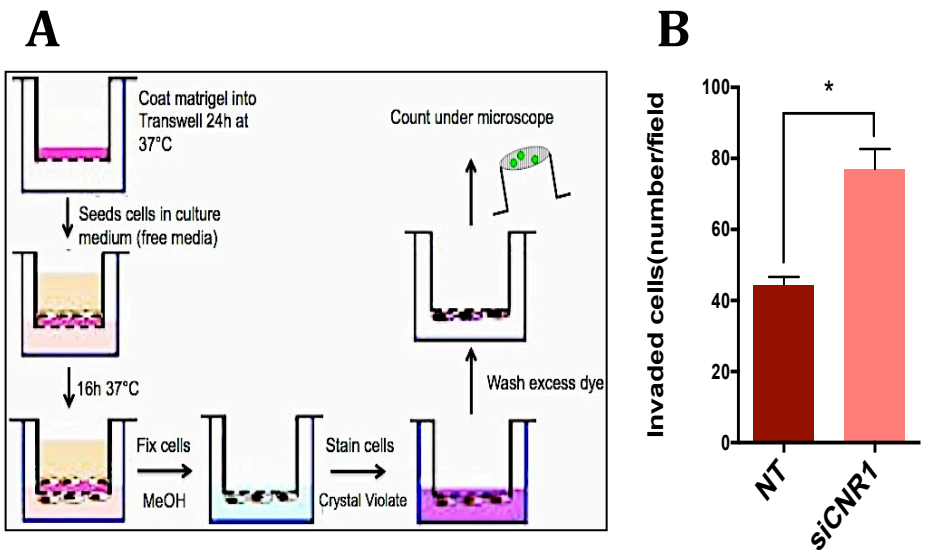


Figure 4.6 The effect of *CNR1* knock-down on cell invasion in DU145 cells examined by Matrigel invasion assay. (A) Schematic representation of Transwell chambers. Transwell chambers consist of a lower and an upper chamber coated with matrigel separated by a porous membrane. **(B)** Bar-chart showing that cells treated with siRNA-CNR1 (48 h) had increased migration to the lower chamber of the Boyden Chambers compared to cells treated with Non-Targeting siRNA (NT). Data are presented as means ± SEM of 2 independent experiments (*P < 0.02). The invasion was measured by determining the number of cells that penetrated through Matrigel-coated Transwell chambers. Cells were counted in five fields.

4.1.4 *KNOCKDOWN OF CNR1 IN DU145 CELLS STIMULATED CELLS IN THE G1-PHASE TO PROGRESS THROUGH THE CELL CYCLE*

To continue investigating the effect of *CNR1* knockdown in DU145 cells, I performed DNA cell cycle analysis to assess the distribution of cells in the cell cycle. Cells ($1.2x10^4$ cells/well) were seeded in 6-well plates and incubated with siCNR1 for 24 h. The siCNR1 treatment resulted in a significant decrease of the number of cells in G1-phase (P> 0.015) of the cell cycle in comparison to non-targeting cells (Figure 4.7) after 24h. The decrease in the number of cells in the G1-phase was coupled with a significant increase in accumulation of cells in the G2/M phases compared to non-targeting cells (P> 0.015). These results suggested that siCNR1 causes cells in G1 to progress and enter the cell cycle promoting cell proliferation (Figure 4.8) by accelerating passage through the cell cycle.

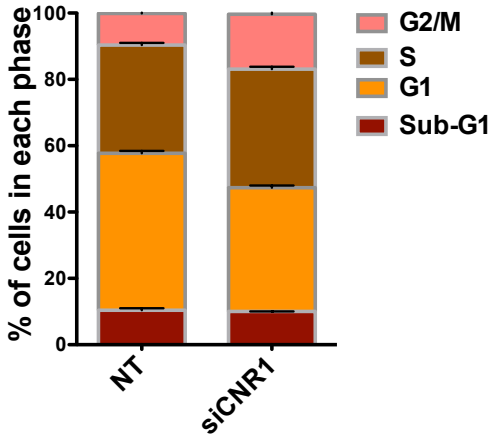
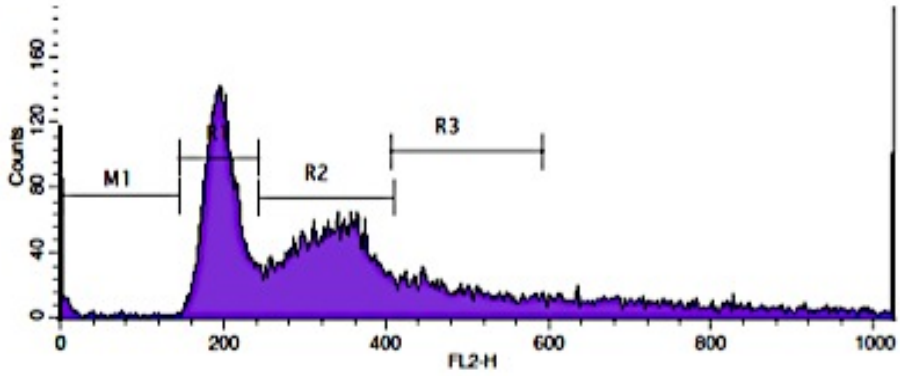


Figure 4.7 CNR1 knockdown effects on cell cycle distribution in DU145 cells. Cells were transfected with siCNR1 and non-targeting reagents for 48 h moved to method section. The siCNR1 treatment resulted in a significant decrease of DU145 cells in G1, increase in S phase (p > 0.05) and G2/M phases (P> 0.015) of the cell cycle in comparison to non-targeting cells. The percentages of cells in the sub-G1, G0/G1, S and G2/M phases were calculated using Waston Pragmatic integration with FlowJo 9.1 software. Data presented here are from two independent experiments.

A Non-targeting DU145 cells



B siCNR1 targeting DU145 cells

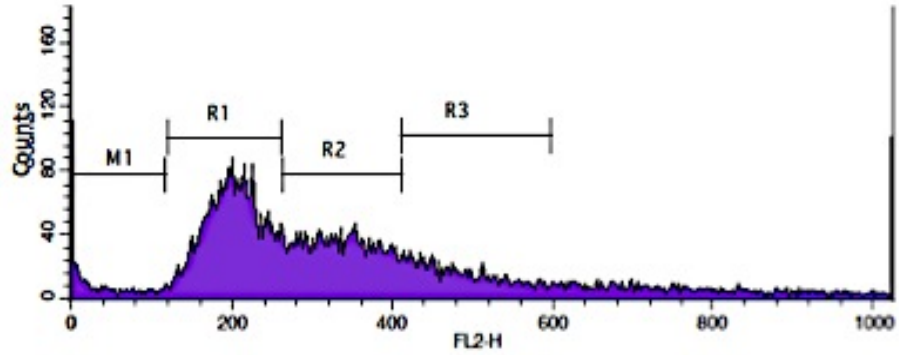


Figure 4.8 Effect of CNR1 knock-down on cell cycle distribution in the DU145 prostate cancer cells. A) Cells were treated with non-targeting siRNA. B) Cells were treated with siRNA-CNR1 reagent. Data shown here are representative of two independent experiments. M1 (sub-G1), R1 (G1), R2 (S), and R3 (G2/M) phases.

4.2 CNR1 ACTIVATION *IN VITRO* TO INVESTIGATE THERAPEUTIC POTENTIAL OF CANNABINOID ANALOGUES IN TREATING PROSTATE CANCER

4.2.1 CNR1 ACTIVATION BY CANNABINOID AGONIST HU210 REDUCES PROSTATE CANCER CELL VIABILITY

Although recent studies have shown that cannabinoids have an inhibitory effect on cell growth and migration of cancer cell (Carracedo, Gironella et al. 2006, Ramer and Hinz 2008, Olea-Herrero, Vara et al. 2009, Guindon and Hohmann 2011, Velasco, Sanchez et al. 2012), not much is known about the effects of cannabinoid agonists on inhibiting prostate cancer growth and progression. I therefore, investigated the effects of the synthetic CNR1 receptor agonist HU210 on prostate cancer cell proliferation, viability and migration.

The HU210 [(−)-1,1-dimethylheptyl is a synthetic CNR1 agonist and an analogue of 11-hydroxy-Δ8–tetrahydrocannabinol. (THC). HU-210 also has affinities for CNR1 and CNR2 receptors that exceed other cannabinoids. Therefore, HU-210 is a particularly potent CNR1 agonist. Its pharmacological effects *in vivo* are also exceptionally long lasting (Pertwee, Howlett et al. 2010). The enhanced affinity and relative intrinsic activity shown by HU-210 at cannabinoid receptors can be largely attributed to the replacement of the pentyl side chain of 8-THC with a dimethylheptyl group, it is a potent CNR1 synthetic agonist (Pertwee 2000, Gilgun-Sherki, Melamed et al. 2003). To my knowledge, the response to HU210 treatment has not been studied in depth in prostate cancer cells.

I observed a dose-independent inhibitory effect of HU210 on the proliferation of all tested prostate cancer cell lines (Figure 4.9) the CNR1-positive LNCaP, 22RV1 and DU145 cells and the low level of CNR1 in PC3 cells. Cells were treated with different concentrations of HU210 (1000, 500, 250, 125, 62.5,

31.25, 15.62, 7.8, 3.9, 1.95 μM) for 24 h and EC_{50}-values were determined by the MTS viability assay (22RV1: 7.2±1.84 μM, LNCaP: 21.9± 5.98 μM, DU145: 29.7± 2.51 μM and PC3: 17.4± 5.88 μM, averages ± SEM; n=3). Cell exposure to HU210 caused dose-dependent cell death in all four prostate cancer cell lines (Figure 4.9A). Furthermore, the treatment with HU210 agonist showed that 22RV1 cells were more sensitive to the agonist compare to other cell lines. Surprisingly, HU210 treatment of PC3 cells resulted in similar EC_{50}-value as in LNCaP cells despite the very low levels of CNR1 protein in PC3 cells (Figure 3.6), suggesting that this small amount of CNR1 receptors are sufficient in suppressing cell growth if activated. Therefore, this CNR1 agonist, such as HU210, can induce cell death in prostate cancer cells.

(R)-SLV 319 also called Ibipinabant, a drug used in clinical research, acts as a potent CNR1 and highly selective CNR1 antagonist with lower lipophilicity. It has been used in scientific research as a drug called (SLV319) (Srivastava, Soni et al. 2008). The effect of (R)-SLV-319 on prostate cancer cell viability is shown in Figure 4.10. Cells were treated with different concentrations of (R)-SLV-319 or DMSO and analysed by the MTS viability assay 24h later. The highest dose of (R)-SLV-319 at 100μM killed <20% of 22RV1, LNCaP and PC3 cells while 60% of DU145 cells were killed at this dose (Figure 4.10). These results demonstrate that the antagonist had no significant effect on cell viability at low doses (<30μM) in any PCa cell line after 24h treatment.

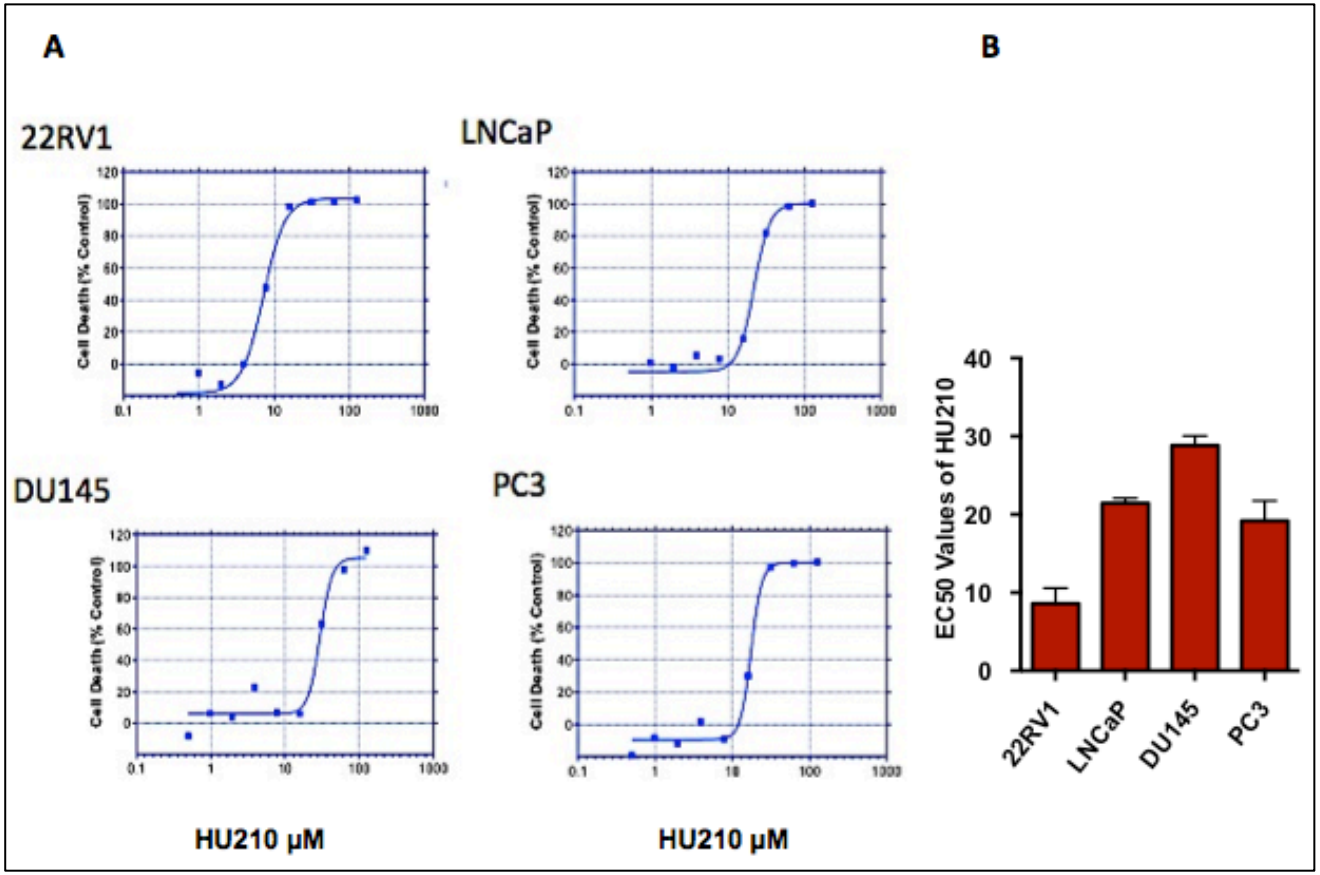


Figure 4.9 Effect of HU210 on prostate cancer cell viability. (**A)** Cell viability was assessed after 24h treatments using the MTS assay to quantify live cells as an indirect measurement of cell death. Cells were treated with different concentrations of HU210 (µM) or DMSO for 24 h (**B)** EC_{50}-values of CNR1 agonist HU210 in µM, see text for values. The percentage of viable cells was determined as the ratio of treated cells to untreated controls (basal or DMSO control). Results are representative of triplicate experiments.

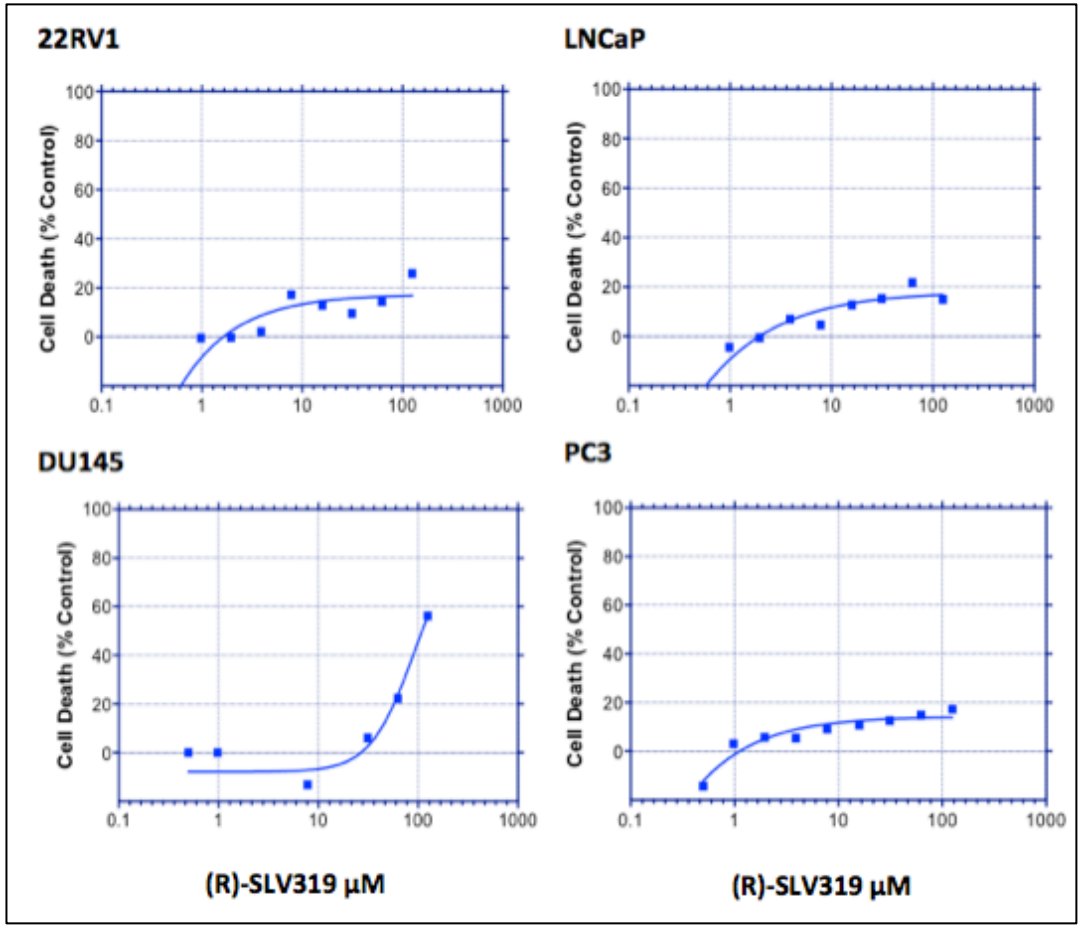


Figure 4.10 Effect of (R)-SLV-319 on prostate cancer cell viability. Cells were treated with different concentrations of (R)-SLV-319 or DMSO for 24 h. Cell viability was assessed using the MTS assay to quantify live cells as an indirect measurement of cell death**.** Results are representative of triplicate experiments.

4.2.2 *HU210-INDUCED CELL KILLING IS MEDIATED THROUGH A CNR1- DEPENDENT MECHANISM*

One of the important aspects of an effective anti-tumour drug is its ability to inhibit proliferation of cancer cells. Cancer cells proliferate rapidly and uncontrolled. Also, cancer cells escape death mechanisms which a normal cell undergoes including apoptosis (Chakravarti, Ravi et al. 2014). Cannabinoids have been demonstrated to be anti-proliferative and induce apoptotic death in cancer cells.

To verify whether the cell death induced in the four prostate cancer cell lines was mediated through specific CNR1 activation, I treated LNCaP, 22RV1, DU145 and PC3 cells for 24 h with a combination of the CNR1 agonist HU210 (25, 10, 30 and 25 μM respectively) based on the EC_{50}-values obtained previously for HU210. The doses of the antagonist (R)-SLV-319 were selected, based on the MTS viability assay (Figure 4.10), at a concentration killing <20% of cells (LNCaP; 25 μM, 22RV!; 15 μM, DU145; 25 μM and PC3; 25 μM). The reduction in cell viability induced by the cannabinoid HU210 was significantly prevented by (R)-SLV-319 in all evaluated cell lines ($P < 0.01$; Figure 4.11). These results indicate that the inhibiting effects of the agonist on cell viability are mediated by CNR1 activation. Furthermore, no significant reduction in cell viability was noted when cells were treated with antagonist alone. The reduction of HU210-induced cell death by (R)-SLV-319 blockage of CNR1 in PC3 cells, which is similar to the value observed in LNCaP cells further confirms that there are low level receptors present in PC3 cells and that this small amount of CNR1 are very active in suppressing cell growth if activated.

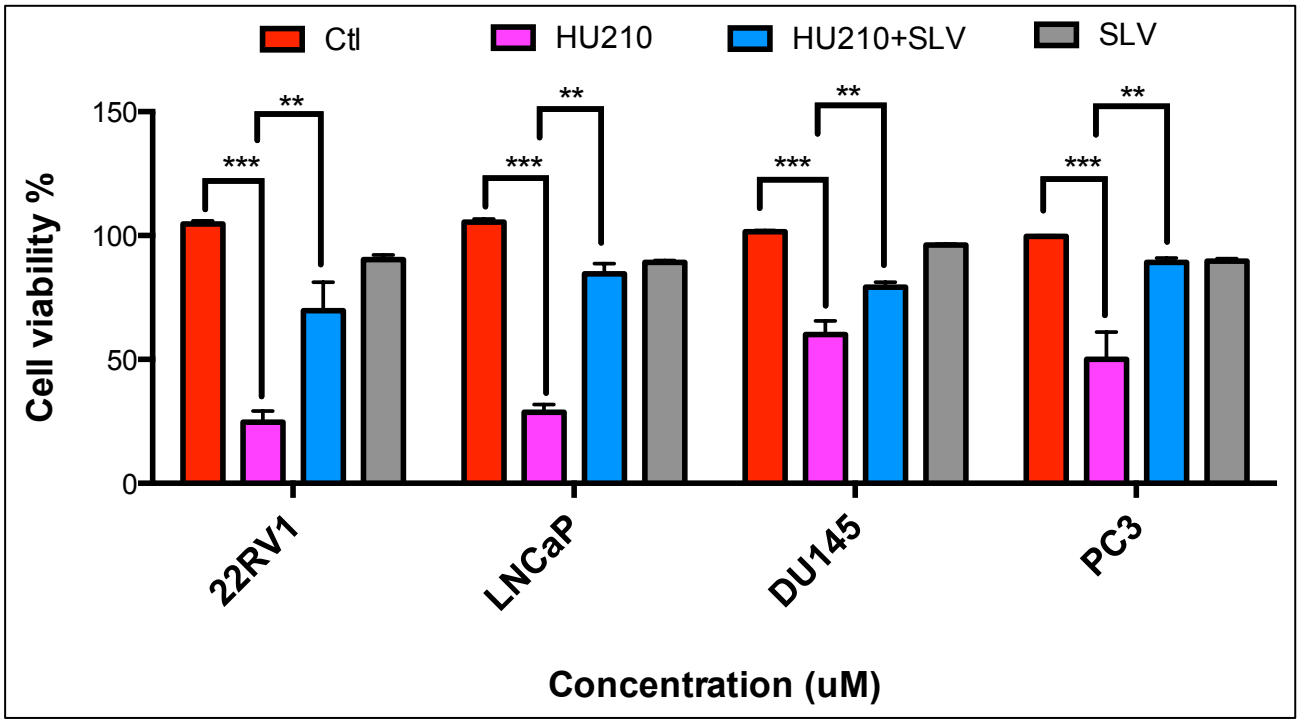


Figure 4.11 HU210 reduces cell viability of 22RV1, LNCaP, DU145 and PC3 cells by activation of the CNR1. 22RV1, LNCaP, DU145 and PC3 cells cultured in serum-free medium in the presence of a combination of CNR1 agonist (HU210) and antagonist (R)-SLV-319) 10/15, 25/25, ,30/25 and 25/25 μM respectively for 24 h. Cell viability decreased in the presence of HU210 that was significantly reversed by addition of (R)-SLV-319. A significant effect on cell viability was not seen in cells treated by the vehicle alone. Cell viability was expressed as a percentage compared to non-treated controls (Ctl). Data are presented as means ± SD of 3 independent experiments (**P < 0.01, ***P < 0.001)

4.2.3 *Evaluation of the induction of apoptosis upon HU210-activated CNR1*

Since treatment of prostate cancer cells lines with HU210 decreased cell viability (section 4.2.1), I therefore, evaluated whether CNR1 activation by HU210 induced apoptosis. Cell cycle analysis was performed by FACS in LNCaP, 22RV1, DU145 and PC3 cells treated with 25, 10, 30 and 25 μM of HU210 for 24 h. DNA content was assessed according to the detection of Propidium Iodide. For all evaluated cell lines, cell cycle histograms showed that HU210 treatment increased the number of cells distributed in the sub-G1 phase in comparison to the untreated cells (Figure 4.12 & 4.13). In PC3 cells, where CNR1 expression is low, HU210 treatment also caused accumulation of 10% of PC3 cells in the sub-G1 phase. These results suggest that HU210 may cause cell death through induction of apoptosis.

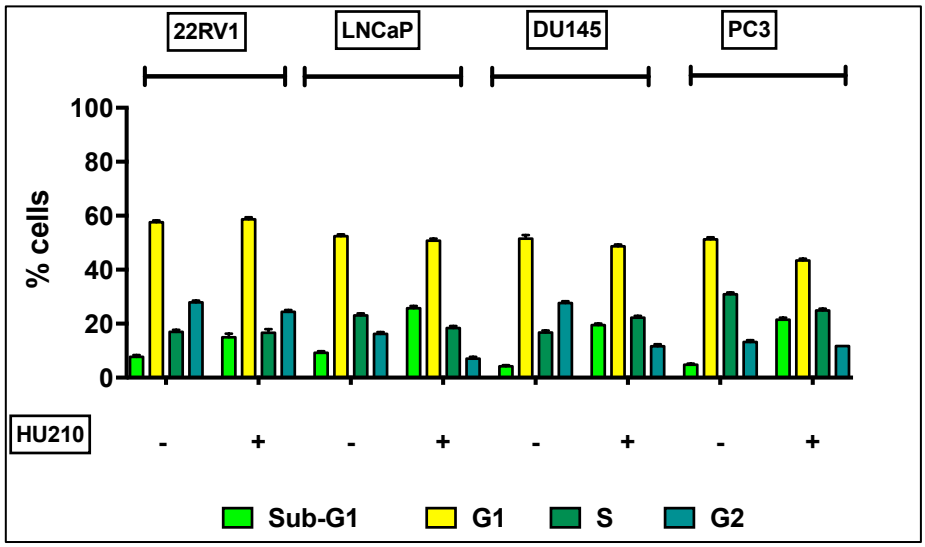


Figure 4.12 The effect of HU210 agonist on cell cycle distribution and sub-G_1 population. The chart indicates the cell cycle phases sub-G1 (apoptosis), G1, S, and G2/M, measured by fluorescence emission at 617 nm (FL-2) after treating with RNase A and staining with PI. The cells treated with HU210 or DMSO for 24 h. The percentage of cells in the sub-G1, G1, S, and G2-M phases was calculated using Waston Pragmatic integration with FlowJo9.1 software. The data shown here are from three independent experiments. The effect of HU210 on inducing apoptosis in prostate cancer cell lines, 22RV1, LNCaP,, DU145 and PC3 was determined by the accumulation of cells in sub-G1 phase.

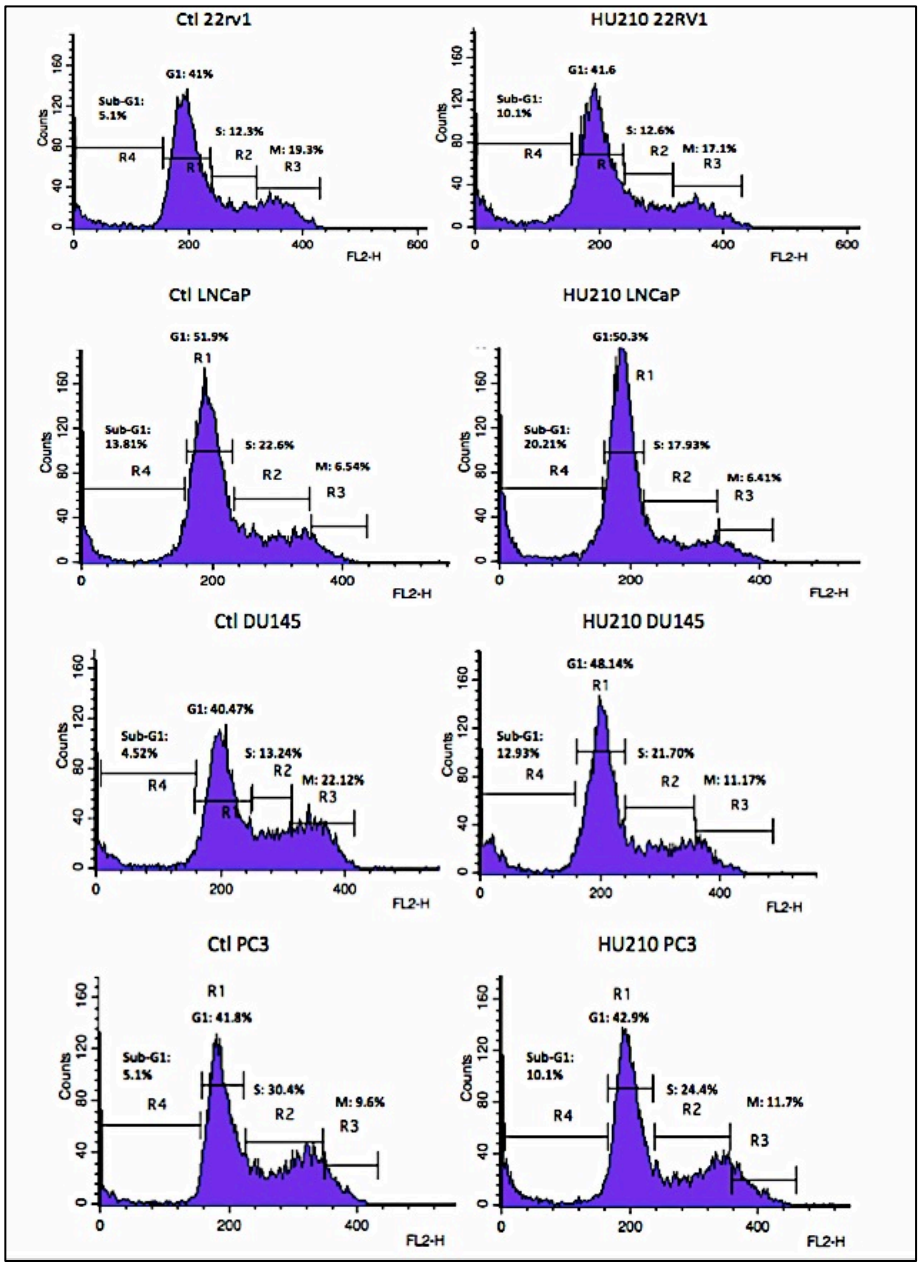


Figure 4.13 HU210 agonist affects cell cycle distribution and the sub-G_1 phase. Representative cell cycle profiles from flow cytometry of 22RV1, LNCaP, DU145 and PC3 cells treated with HU210 or DMSO for 24 h. Data shown here are representative of three independent experiments. The histogram indicates the phases of the cell cycle: sub-G1, G1, S and G2/M.

To further investigate whether apoptosis played a role in the HU210-mediated cell death, I explored the levels of caspase-3 activation and PARP cleavage by western blot. Cells were treated with 4 μM HU210 for 1 h and cell lysates analysed for cleavage of PARP and pro-caspase-3 after 24h. I found that HU210 treatment decreased the levels of procaspase-3 in all prostate cancer cell lines. the downstream signals during apoptosis are transmitted via caspases, which upon conversion from inactive to active cleaved forms mediate the cleavage of PARP. I found HU210 treatment caused increased cleavage of the 116 kDa PARP protein to the 89 kDa form that is characteristic for apoptosis (Figure 4.14) in all prostate cancer cell lines except in 22RV1 cells. Protein intensity revealed a decrease in the protein expression of PARP protein (116 kDa) with a concomitant increase in its cleaved product (89 kDa).

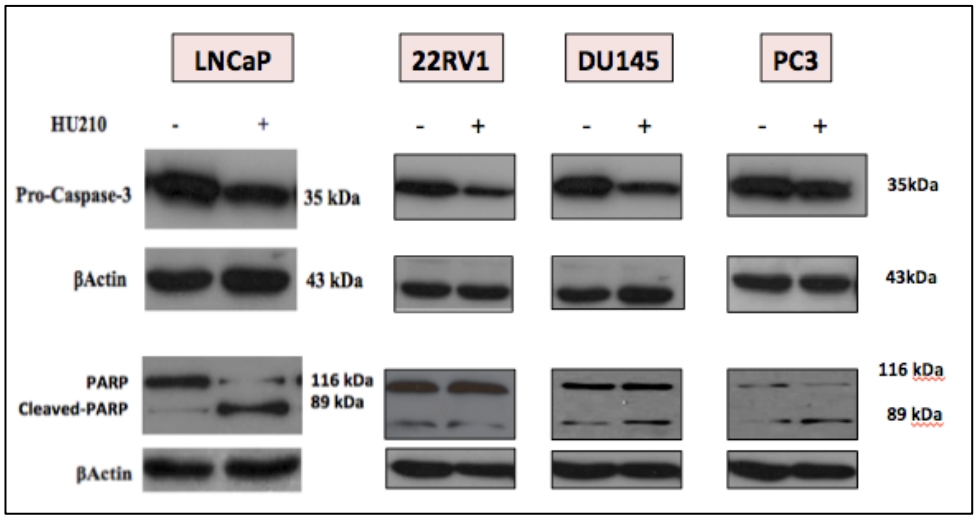


Figure 4.14 Effect of HU210 treatment on pro-caspase-3 and PARP protein expression. The protein expression of procaspase-3, PARP and cleavage of PARP in prostate cancer cell lines. As detailed in methods, the cells were treated with HU210 or DMSO alone for 1h and total cell lysates were prepared for western blot analysis. β-actin was used as a loading control with expected size at 43 kDa. The data shown here are from a representative experiment repeated two times with similar results.

4.3 DISCUSSION

4.3.1 EFFECT OF CNR1 KNOCKDOWN IN PROSTATE CANCER CELLS

Cancer cells carry the tumour suppressor and oncogene mutations that define cancer as a genetic disease (Hanahan and Weinberg). TSGs function by one of the following mechanisms: DNA repair, inhibition of regulated progression through cell cycle, induction of apoptosis and inhibition of cellular migration and metastasis.

The altered CNR1 expression has been linked with various cancers (Bedoya, Meneu et al. 2009, Bedoya, Rubio et al. 2009, Larrinaga, Begoaa et al. 2010, Meneu-Diaz, Bedoya et al. 2011). However, the exact role of CNR1 in prostate cancer proliferation has not been clarified. To my knowledge, the response to CNR1 knockdown had not been previously investigated in prostate cancer. Therefore, I investigated the role of CNR1 in prostate cancer by knocking-down the receptor in the prostate cancer cell line DU145, and examined whether the knockdown would increase cell viability. I found that depletion of CNR1 led to a significant increases in cell proliferation and colony formation. Similar results have been reported in colorectal cancer where the inhibition of CNR1 accelerated intestinal adenoma growth in $Apc^{Min/+}$ mice whereas activation of CNR1 attenuated intestinal tumour growth by inducing cell death via down-regulation of anti-apoptotic factor survivin (Wang, Wang et al. 2008). These results suggest that activation of the CNR1 gene may inhibit cell proliferation in prostate cancer cells. It would be interesting to further investigate if survivin may also play a role in mediating CNR1 mediated prostate cancer cell growth inhibition.

Metastasis is a multifactorial process that includes the acquisition of a motile and invasive phenotype. During progression to a metastatic phenotype, tumour cells undergo a series of changes that begin with loss of contact inhibition and increased motility, allowing them to migrate from the primary tumour site, invade distant organs, and induce neo-vascularization resulting in metastasis (Laezza, Pisanti et al. 2008). Several models of tumour invasion and metastasis have been

proposed, although different mechanisms will be used in different cancers (Weigelt, Peterse et al. 2005, Phay and Ringel 2013). In my study I found that knocking down CNR1 increased the ability of DU145 cells to invade a Matri-gel matrix. It has been reported that CNR1 receptor mediates inhibition of transmigration in MDA-MB-231, a highly invasive human breast cancer cell line (Laezza, Pisanti et al. 2008). My study demonstrates that CNR1 can inhibit the growth and invasion of prostate cancer cells and further support the involvement of CNR1 inactivation in the malignant progression of prostate cancer. Therefore, the above results provided evidence to suggest that CNR1 may play a potential tumour suppressor role in prostate cancer.

It is well recognised that uncontrolled cellular growth, a consequence of defects in the cell cycle, is responsible for the development of most cancers including prostate cancer (Sarfaraz, Afaq et al. 2006). I therefore, further investigate the effect of CNR1 as a TSG on the cell cycle distribution in prostate cancer cell lines. In this part of my research, I found that CNR1 knockdown resulted in significant decrease of DU145 cells in the G1-phase of the cell cycle in comparison to non-targeting cells. The knockdown of CNR1 stimulates the cells from G1 to enter the cell cycle resulting in cell proliferation. It would be interesting to further investigate cell migration and invasion in the presence of agonist and antagonist to prove the activation of CNR1 may suppress tumour progression by inhibiting cancer cell migration and invasion .

4.3.2 CNR1 ACTIVATION IN VITRO TO INVESTIGATE THERAPEUTIC POTENTIAL OF CANNABINOID ANALOGUES IN TREATING PROSTATE CANCER

Endocannabinoids and other CNR1 agonists are drawing renewed attention because of their diverse pharmacological activities such as cell growth inhibition, anti-inflammatory effects, and tumour regression in many cancers (Freimuth, Ramer et al. 2010, Ramos and Bianco 2012). Data from *in vitro* and *in vivo* experiments suggest that cannabinoid receptor

agonists can reduce tumour growth and induce apoptosis in several tumour types, including melanoma, breast and prostate cancer, colon cancer, leukemia, and glioma (Joseph, Niggemann et al. 2004, Grimaldi, Pisanti et al. 2006, Chakravarti, Ravi et al. 2014, Javid, Phillips et al. 2016). Hu210 inhibit the proliferation of EFM-19 breast cancer cells (De Petrocellis, Melck et al. 1998, Melck, De Petrocellis et al. 2000). In addition, Cannabinoids have been shown to prevent cell proliferation and induce apoptosis in prostate cancer (Carracedo, Gironella et al. 2006, Sarfaraz, Afaq et al. 2006, Preet, Qamri et al. 2011, Ellert-Miklaszewska, Ciechomska et al. 2013, Pellerito, Notaro et al. 2014, Orellana-Serradell, Poblete et al. 2015). However, the response to HU210 cannabinoid treatment and its role in the control of tumour proliferation has not been studied in depth in prostate cancer. In this part of my study, I investigated the effects of cannabinoid receptor agonists (HU210) in the prostate cancer cell lines and demonstrated that the cannabinoid receptor agonist HU210 exerted an antiproliferative and proapoptotic action on prostate cancer cell lines through activation of the CNR1 receptor. I found that treatment with the CNR1 agonist HU210 of the prostate cancer cells (22RV1, LNCaP, DU145 and PC3) resulted in dose-dependent decreases of cell viability as determined by MTS assay. The specificity of this effect for CNR1 was supported by the CNR1-specific antagonist (R)-SLV-319, which was able to significantly restore cell viability in HU210 treated cells. Surprisingly, HU210 treatment also reduced cell viability of the PC3 cells, despite the low levels of CNR1 protein in PC3 cells, suggesting that there are low level receptors present and that this small amount of CNR1 is sufficient in suppressing cell growth if activated. It has been reported in one study where HU210 rapidly blocked PC3 cell viability and halted cell mobility (Christopher John Fowler 2009). Therefore, the use of the specific CNR1 receptor blocker (R)-SLV-319 confirmed that this effect was produced primarily from the activation of the CB1 receptor. Although there may be other pathways causing HU210 induced anti-proliferative effects in PC3 cells, the inhibition of HU210 induced anti-proliferative effects by (R)-SLV-319 confirms that HU210 induced anti-proliferative effects is mediated by activation of this small amount of CNR1 in PC3. It would be interesting to investigate further other pathways that may trigger the activation of CNR1 in PC3 cells, which may have the potential to decrease cancer development, growth, and metastasis.

As cancer cells not only proliferate rapidly and uncontrollably but also escape apoptosis, a programmed cell death mechanism that involves the activation of caspase dependent and independent pathways (Hanahan and Weinberg). When the balance between proliferation and apoptosis is lost tumour progression may occur. Consistent with this concept, there is a need to identify novel targets and mechanism-based apoptosis-inducing agents for the management of prostate cancer. It is generally recognised that apoptosis is a pivotal cellular mechanism to eliminate cancer cells. I therefore, evaluated whether the treatment with the cannabinoid receptor agonist HU210 lowers the viability of the prostate cancer cells (22RV1, LNCaP, DU145 and PC3) through the induction of apoptosis. Using western blot analysis, I observed that HU210 treatment caused a significant decrease in the inactive procaspase-3, suggesting cleavage to active caspase-3. In agreement with caspase-3 activation the 116 kDa PARP protein was cleaved into its apoptosis-related 89 kDa product, which is consistent with the effect of HU210 treatment on the accumulation of cells in the sub-G1 phase in all tested cell lines. This is interesting and may be useful for designing strategies for the management of human prostate cancer because CNR1 agonists could reactivate apoptosis. Surprisingly, I did not observe cleaved caspase-3; the antibody that I used should detect both pro-caspase (35 kDa) and cleaved caspase-3 (17 kDa & 12 kDa). I checked if there were low molecular weight proteins in the membranes after staining with Ponceu. Unfortunately, no weak signal for the cleaved caspase-3. It is possible that there is a technical issue to sensitively detect cleaved caspase-3.

Based on all these data, there is a potential to target CNR1 for prostate cancer therapy. It is may be feasible to develop a cannabinoid receptor agonists such as HU210 into a novel therapeutic agent for the treatment of prostate cancer.

5 Concluding Remarks

More research is necessary to substantiate the suppressor role of *CNR1* in the management of prostate cancer. One potential strategy to progress

the state of knowledge and expertise in the area of prostate cancer treatment is presented by the current investigation that demonstrated the potential role of the *CNR*1 gene in prostate cancer. The results of this thesis provide additional evidence that *CNR1* gene may have a tumour-suppressing role in prostate cells.

In order to study the tumour-suppressor role of this gene in prostate cancer, I investigated the potential mutations in the coding and promoter regions of the *CNR1* gene in several prostate cancer and non-malignant prostate cell lines and 73 prostate cancer samples with combined fluidigm amplification and next generation sequencing and found the absence of *CNR1* mutation. This means that although *CNR1* mutation can be a genetic mechanism for its inactivation in other cancer development (Wang, Wang et al. 2008, Bedoya, Meneu et al. 2009, Bedoya, Rubio et al. 2009), it did not appear to contribute to *CNR1* inactivation in the tested prostate cancer samples. Therefore. 6q15 region genomic deletion, which occurs in nearly half of prostate cancer, may be the main mechanism that leads to the loss of function of *CNR1* in prostate cancer. Recently, epigenetic modification, in particular DNA methylation, has been shown playing an important role in cancer development and progression. It should be further investigated if and to what extend DNA methylation contributes to loss of *CNR1* function.

I found a high frequency of the longer *CNR1* allele of the 6 bp polymorphism in the promoter region in prostate cancer cell lines, with the potential to bind additional transcription factor proteins. However, our further investigation of the polymorphism in a Chinese prostate cancer case control study did not support a role in prostate carcinogenesis in the Chinese population. As the polymorphism in the *CNR1* promoter has a trend to correlate to *CNR1* expression levels in the limited number of prostate cancer cell lines that were available, further studies will be required to investigate if this polymorphism disrupts the transcription activity of *CNR1* and contributes to prostate carcinogenesis in the European population.

Moving from genomic studies to transcript and protein expression analysis, *CNR1* has been shown to be expressed at the mRNA and protein levels in the prostate cancer cell lines 22RV1, LNCaP, DU145 and PC3 by using qRT-PCR and Western blotting, respectively. Moreover, in order to confirm and clarify its tumour-suppressing role, *CNR1* was further studied at the cellular level by diverse functional assays and cell cycle analysis in *CNR1* knocked-down DU145 cells. I observed that abrogation of *CNR1* activity in these cells resulted in increased cell viability, migration and invasion. These cellular studies suggested that *CNR1* might act as a tumour suppressor gene in prostate cancer.

Another interesting finding is that *CNR1* knockdown causes cell proliferation via cell cycle progression. This is the first reported study on knockdown of *CNR1* in prostate cancer suggesting that *CNR1* may act as a tumour suppressor gene in prostate cells. Our findings provide another new evidence that *CNR1* plays an essential role in cell growth and may be a potent therapeutic target in human prostate cancer, based on which we could develop more effective therapeutic approaches to prolong patient survival.

Therefore, I determined the effectiveness of using cannabinoid agonists for the treatment of prostate cancer by using the CNR1 agonist HU210 in 22RV1, LNCaP, DU145 and PC3 cells. Treatment of 22RV1, LNCaP, DU145, and PC3 cells with HU210 significantly decreased cell viability, indicating the anti-proliferative effects of HU210 via activation of the cannabinoid receptors. Furthermore, the CNR1-specific antagonist (R)-SLV-319 was able to significantly restore cell viability suggesting that inhibiting effects of the agonist on cell viability are mediated by CNR1 activation. Moreover, caspase-3 protein expression evaluated by Western blotting revealed that treatment with HU120 activated the apoptotic pathways in prostate cancer cell lines. Our findings thus demonstrate that HU210 is a promising CNR1 agonist agent to use to inhibit prostate cancer

cell growth via CNR1 receptors for potential anti-cancer treatment in patients.

While the anti-tumourigenic effects of the CNR1 ligands such as endocannabinoids and CNR1 agonists may be used for the treatment of prostate cancer patients by affecting cancer cell growth, apoptosis, angiogenesis, migration, invasion, adhesion, and formation of metastasis (Freimuth, Ramer et al. 2010), the tumour suppressor role of *CNR1* as demonstrated by the current study also suggests the potential use of exogenous *CNR1* gene in gene therapy. CNR1 gene therapy may be especially applicable in patients with tumours expressing low or no *CNR1*. Other research streams could target the down-stream gene(s) of *CNR1* pathway(s) (Freimuth, Ramer et al. 2010, Fowler, Josefsson et al. 2013).

All together, these results demonstrate the foremost tumour-suppressing role of *CNR1* gene by influencing the proliferation, migration and invasion of prostate cancer. Nonetheless these results could encourage further investigation of the role of the *CNR1* gene in prostate cancer, and lead to novel promising treatment strategies in prostate cancer.

6 FUTURE PLANS

6.1 CHARACTERISATION OF CNR1 STATUS IN PROSTATE CANCER

6.1.1 The effect of the polymorphism in *CNR1* promoter regions in gene transcription activity

We have confirmed that the *CNR1* gene has a variant, rs147446147 insertion located within the upstream promoter region. Given that this variant is located in the promoter region, it may affect gene activity and transcription. The effect of the variants on *CNR1* gene expression will be investigated with relation to prostate cancer. Both the longer and shorter allele DNA fragment of the upstream region of the *CNR1* gene will be subcloned into the promoter region of a luciferase reporter system for analysing the promoter activity with or without the rs147446147 6 bp sequence.

6.1.2 DNA methylation in CNR1 in prostate cancer

Inactivation of tumour suppressor genes in cancer cells resulting from epigenetic silencing, such as DNA methylation, is as important as genomic inactivation. Therefore, whether DNA methylation of *CNR1* contributes to the low levels of transcription will be examined in PC3 cells. These cells will be treated with the demethylating agent 5-aza-dC and then *CNR1* mRNA and protein expression will be analysed. The methylation of the *CNR1* upstream promoter sequence will be analysed (1kbp) in prostate cancer samples using Bisulfate genomic sequencing.

6.2 STUDY OF THE ROLE OF *CNR1* IN PROSTATE CANCER

Since CNR1 agonist HU210 triggers apoptosis in LNCaP cells, this same effect will be determined in other prostate cancer cell lines, and whether this effect correlates with the endogenous levels of CNR1 expression. It will also be investigated whether increasing the CNR1 expression affects responses to agonist treatment in prostate cancer cell lines with low endogenous levels of CNR1 expression (e.g. PC3 cells). Finally, it will be investigated whether this effect is specific to this CNR1 agonist and the effect of CNR1 antagonists, (which could inhibit apoptosis).

The collected data show that CNR1 agonist (HU210) can induce growth inhibition and cell death. Classical caspase-dependent pathway will be analysed for its involvment in inducing apoptosis in prostate cancer cell line using apoptosis assays such as FITC annexin V apoptosis detection kits and detection of caspase activation (cleavage). Also, most cancer treatments consist of multiple drugs working together to eliminate the malignant cells. It would be interesting to study combinations of cannabinoids (HU210 agonist) with therapeutic drugs.

Most of the drugs that inhibit the growth of cancer cells *in vitro* turn out to be ineffective when tested in animals. However, there is now evidence that few cannabinoids activate cannabinoid receptors may act as apoptotic drugs *in vivo*. For example, THC administration can effectively reduce the growth of gliomas in mice by inducing apoptosis of the tumour cells (Galve-Roperh, Sanchez et al. 2000). Moreover, previous studies showed that HU210 is capable of reducing rhabdomyosarcoma (aRMS) xenograft growth through induction of apoptosis *in vivo* where proliferation of tumour cells remained unaffected. It would be interesting to investigate the role of CNR1 in PCa tumour growth xenograft studies *in vivo* and determine efficacy and toxicity after administration of HU210.

To further investigate the role of *CNR1* in cell survival and proliferation, CNR1 will be overexpressed in different prostate cell lines, and a series

of functional assays will be performed, including cell viability (MTS), apoptosis and cell cycle assays. Also invasion and migration assays will be performed to determine whether CNR1 affects cell motility. Soft agar colony formation assay will be performed to investigate whether *CNR1* expression affects cell anchor-independent growth.

It has been reported that treatment of gliomas with cannabinoids led to the activation of ERK1/2 signalling pathway and AKT inhibition (Ellert-Miklaszewska, Ciechomska et al. 2013). This continued ERK1/2 activation could mediate cell-cycle arrest and apoptosis. I will investigate the effect of *CNR1* agonists on the activation of ERK and the inhibition of PI3K/AKT in prostate cancer, in order to understand the mechanism by which apoptosis is triggered in response to CNR1 receptor activation.

7 REFERENCES

Abate-Shen, C., M. M. Shen and E. Gelmann (2008). "Integrating differentiation and cancer: The Nkx3.1 homeobox gene in prostate organogenesis and carcinogenesis." Differentiation **76**(6): 717-727.
Akamatsu, S., R. Takata, C. A. Haiman, A. Takahashi, T. Inoue, M. Kubo, M. Furihata, N. Kamatani, J. Inazawa, G. K. Chen, L. Le Marchand, L. N. Kolonel, T. Katoh, Y. Yamano, M. Yamakado, H. Takahashi, H. Yamada, S. Egawa, T. Fujioka, B. E. Henderson, T. Habuchi, O. Ogawa, Y. Nakamura and H. Nakagawa (2012). "Common variants at 11q12, 10q26 and 3p11.2 are associated with prostate cancer susceptibility in Japanese." Nature genetics **44**(4): 426-429.
Al Olama, A. A., Z. Kote-Jarai, S. I. Berndt, D. V. Conti, F. Schumacher, Y. Han, S. Benlloch, D. J. Hazelett, Z. Wang, E. Saunders, D. Leongamornlert, S. Lindstrom, S. Jugurnauth-Little, T. Dadaev, M. Tymrakiewicz, D. O. Stram, K. Rand, P. Wan, A. Stram, X. Sheng, L. C. Pooler, K. Park, L. Xia, J. Tyrer, L. N. Kolonel, L. Le Marchand, R. N. Hoover, M. J. Machiela, M. Yeager, L. Burdette, C. C. Chung, A. Hutchinson, K. Yu, C. Goh, M. Ahmed, K. Govindasami, M. Guy, T. L. J. Tammela, A. Auvinen, T. Wahlfors, J. Schleutker, T. Visakorpi, K. A. Leinonen, J. Xu, M. Aly, J. Donovan, R. C. Travis, T. J. Key, A. Siddiq, F. Canzian, K.-T. Khaw, A. Takahashi, M. Kubo, P. Pharoah, N. Pashayan, M. Weischer, B. G. Nordestgaard, S. F. Nielsen, P. Klarskov, M. A. R√∏der, P. Iversen, S. N. Thibodeau, S. K. McDonnell, D. J. Schaid, J. L. Stanford, S. Kolb, S. Holt, B. Knudsen, A. H. Coll, S. M. Gapstur, W. R. Diver, V. L. Stevens, C. Maier, M. Luedeke, K. Herkommer, A. E. Rinckleb, S. S. Strom, C. Pettaway, E. D. Yeboah, Y. Tettey, R. B. Biritwum, A. A. Adjei, E. Tay, A. Truelove, S. Niwa, A. P. Chokkalingam, L. Cannon-Albright, C. Cybulski, D. Woko≈Çorczyk, W. Klu≈ ∫ niak, J. Park, T. Sellers, H.-Y. Lin, W. B. Isaacs, A. W. Partin, H. Brenner, A. K. Dieffenbach, C. Stegmaier, C. Chen, E. L. Giovannucci, J. Ma, M. Stampfer, K. L. Penney, L. Mucci, E. M. John, S. A. Ingles, R. A. Kittles, A. B. Murphy, H. Pandha, A. Michael, A. M. Kierzek, W. Blot, L. B. Signorello, W. Zheng, D. Albanes, J. Virtamo, S. Weinstein, B. Nemesure, J. Carpten, C. Leske, S.-Y. Wu, A. Hennis, A. S. Kibel, B. A. Rybicki, C. Neslund-Dudas, A. W. Hsing, L. Chu, P. J. Goodman, E. A. Klein, S. L. Zheng, J. Batra, J. Clements, A. Spurdle, M. R. Teixeira, P. Paulo, S. Maia, C. Slavov, R. Kaneva, V. Mitev, J. S. Witte, G. Casey, E. M. Gillanders, D. Seminara, E. Riboli, F. C. Hamdy, G. A. Coetzee, Q. Li, M. L. Freedman, D. J. Hunter, K. Muir, H. Gronberg, D. E. Neal, M. Southey, G. G. Giles, G. Severi, B. The, C. Prostate Cancer Cohort, P. C. The, C. C. The, G.-O. N. E. C. The, M. B. Cook, H. Nakagawa, F. Wiklund, P. Kraft, S. J. Chanock, B. E. Henderson, D. F. Easton, R. A. Eeles and C. A. Haiman (2014). "A meta-analysis of 87,040 individuals identifies 23 new susceptibility loci for prostate cancer." Nature genetics **46**(10): 1103-1109.
Al Olama, A. A., Z. Kote-Jarai, S. I. Berndt, D. V. Conti, F. Schumacher, Y. Han, S. Benlloch, D. J. Hazelett, Z. Wang, E. Saunders, D. Leongamornlert, S. Lindstrom, S. Jugurnauth-Little, T. Dadaev, M. Tymrakiewicz, D. O. Stram, K. Rand, P. Wan, A. Stram, X. Sheng, L. C. Pooler, K. Park, L. Xia, J. Tyrer, L. N. Kolonel, L. Le Marchand, R. N. Hoover, M. J. Machiela, M. Yeager, L. Burdette, C. C. Chung, A. Hutchinson, K. Yu, C. Goh, M. Ahmed, K. Govindasami, M. Guy, T. L. J. Tammela, A. Auvinen, T. Wahlfors, J. Schleutker, T. Visakorpi, K. A. Leinonen, J. Xu, M. Aly, J. Donovan, R. C. Travis, T. J. Key, A. Siddiq, F. Canzian, K.-T. Khaw, A. Takahashi, M. Kubo, P. Pharoah, N. Pashayan, M. Weischer, B. G. Nordestgaard, S. F. Nielsen, P. Klarskov, M. A. Roder, P. Iversen, S. N. Thibodeau, S. K. McDonnell, D. J. Schaid, J. L. Stanford, S. Kolb, S. Holt, B. Knudsen, A. H. Coll, S. M. Gapstur, W. R. Diver, V. L. Stevens, C. Maier, M. Luedeke, K. Herkommer, A. E. Rinckleb, S. S. Strom, C. Pettaway, E. D.

Yeboah, Y. Tettey, R. B. Biritwum, A. A. Adjei, E. Tay, A. Truelove, S. Niwa, A. P. Chokkalingam, L. Cannon-Albright, C. Cybulski, D. Wokolorczyk, W. Kluzniak, J. Park, T. Sellers, H.-Y. Lin, W. B. Isaacs, A. W. Partin, H. Brenner, A. K. Dieffenbach, C. Stegmaier, C. Chen, E. L. Giovannucci, J. Ma, M. Stampfer, K. L. Penney, L. Mucci, E. M. John, S. A. Ingles, R. A. Kittles, A. B. Murphy, H. Pandha, A. Michael, A. M. Kierzek, W. Blot, L. B. Signorello, W. Zheng, D. Albanes, J. Virtamo, S. Weinstein, B. Nemesure, J. Carpten, C. Leske, S.-Y. Wu, A. Hennis, A. S. Kibel, B. A. Rybicki, C. Neslund-Dudas, A. W. Hsing, L. Chu, P. J. Goodman, E. A. Klein, S. L. Zheng, J. Batra, J. Clements, A. Spurdle, M. R. Teixeira, P. Paulo, S. Maia, C. Slavov, R. Kaneva, V. Mitev, J. S. Witte, G. Casey, E. M. Gillanders, D. Seminara, E. Riboli, F. C. Hamdy, G. A. Coetzee, Q. Li, M. L. Freedman, D. J. Hunter, K. Muir, H. Gronberg, D. E. Neal, M. Southey, G. G. Giles, G. Severi, B. The, C. Prostate Cancer Cohort, P. C. The, C. C. The, G.-O. N. E. C. The, M. B. Cook, H. Nakagawa, F. Wiklund, P. Kraft, S. J. Chanock, B. E. Henderson, D. F. Easton, R. A. Eeles and C. A. Haiman (2014). "A meta-analysis of 87,040 individuals identifies 23 new susceptibility loci for prostate cancer." Nat Genet **46**(10): 1103-1109.

Albany, C., A. S. Alva, A. M. Aparicio, R. Singal, S. Yellapragada, G. Sonpavde and N. M. Hahn (2011). "Epigenetics in Prostate Cancer." Prostate Cancer **2011**: 12.

Albertsen, P. C. (2011). "When is active surveillance the appropriate treatment for prostate cancer?" Acta oncologica **50**(S1): 120-126.

Alexander, A., P. F. Smith and R. J. Rosengren (2009). "Cannabinoids in the treatment of cancer." Cancer letters **285**(1): 6-12.

Amaral, T. M. S., D. Macedo, I. Fernandes and L. Costa (2012). "Castration-Resistant Prostate Cancer: Mechanisms, Targets, and Treatment." Prostate Cancer **2012**: 11.

Amin Al Olama, A., Z. Kote-Jarai, F. R. Schumacher, F. Wiklund, S. I. Berndt, S. Benlloch, G. G. Giles, G. Severi, D. E. Neal, F. C. Hamdy, J. L. Donovan, D. J. Hunter, B. E. Henderson, M. J. Thun, M. Gaziano, E. L. Giovannucci, A. Siddiq, R. C. Travis, D. G. Cox, F. Canzian, E. Riboli, T. J. Key, G. Andriole, D. Albanes, R. B. Hayes, J. Schleutker, A. Auvinen, T. L. J. Tammela, M. Weischer, J. L. Stanford, E. A. Ostrander, C. Cybulski, J. Lubinski, S. N. Thibodeau, D. J. Schaid, K. D. Sorensen, J. Batra, J. A. Clements, S. Chambers, J. Aitken, R. A. Gardiner, C. Maier, W. Vogel, T. D√ ∂ rk, H. Brenner, T. Habuchi, S. Ingles, E. M. John, J. L. Dickinson, L. Cannon-Albright, M. R. Teixeira, R. Kaneva, H.-W. Zhang, Y.-J. Lu, J. Y. Park, K. A. Cooney, K. R. Muir, D. A. Leongamornlert, E. Saunders, M. Tymrakiewicz, N. Mahmud, M. Guy, K. Govindasami, L. T. O'Brien, R. A. Wilkinson, A. L. Hall, E. J. Sawyer, T. Dadaev, J. Morrison, D. P. Dearnaley, A. Horwich, R. A. Huddart, V. S. Khoo, C. C. Parker, N. Van As, C. J. Woodhouse, A. Thompson, T. Dudderidge, C. Ogden, C. S. Cooper, A. Lophatonanon, M. C. Southey, J. L. Hopper, D. English, J. Virtamo, L. Le Marchand, D. Campa, R. Kaaks, S. Lindstrom, W. R. Diver, S. Gapstur, M. Yeager, A. Cox, M. C. Stern, R. Corral, M. Aly, W. Isaacs, J. Adolfsson, J. Xu, S. L. Zheng, T. Wahlfors, K. Taari, P. Kujala, P. Klarskov, B. r. G. Nordestgaard, M. A. R√ ∏der, R. Frikke-Schmidt, S. E. Bojesen, L. M. FitzGerald, S. Kolb, E. M. Kwon, D. M. Karyadi, T. F. Orntoft, M. Borre, A. Rinckleb, M. Luedeke, K. Herkommer, A. Meyer, J. r. Serth, J. R. Marthick, B. Patterson, D. Wokolorczyk, A. Spurdle, F. Lose, S. K. McDonnell, A. D. Joshi, A. Shahabi, P. Pinto, J. Santos, A. Ray, T. A. Sellers, H.-Y. Lin, R. A. Stephenson, C. Teerlink, H. Muller, D. Rothenbacher, N. Tsuchiya, S. Narita, G.-W. Cao, C. Slavov, V. Mitev, U. K. G. P. C. S. C. B. A. o. U. S. S. o. O. The, U. K. P. S. C. The, B. The Australian Prostate Cancer, P. C. The, S. Chanock, H. Gronberg, C. A. Haiman,

P. Kraft, D. F. Easton and R. A. Eeles (2013). "A meta-analysis of genome-wide association studies to identify prostate cancer susceptibility loci associated with aggressive and non-aggressive disease." Human molecular genetics **22**(2): 408-415.
Amundadottir, L. T., P. Sulem, J. Gudmundsson, A. Helgason, A. Baker, B. A. Agnarsson, A. Sigurdsson, K. R. Benediktsdottir, J. B. Cazier, J. Sainz, M. Jakobsdottir, J. Kostic, D. N. Magnusdottir, S. Ghosh, K. Agnarsson, B. Birgisdottir, L. Le Roux, A. Olafsdottir, T. Blondal, M. Andresdottir, O. S. Gretarsdottir, J. T. Bergthorsson, D. Gudbjartsson, A. Gylfason, G. Thorleifsson, A. Manolescu, K. Kristjansson, G. Geirsson, H. Isaksson, J. Douglas, J. E. Johansson, K. Balter, F. Wiklund, J. E. Montie, X. Y. Yu, B. K. Suarez, C. Ober, K. A. Cooney, H. Gronberg, W. J. Catalona, G. V. Einarsson, R. B. Barkardottir, J. R. Gulcher, A. Kong, U. Thorsteinsdottir and K. Stefansson (2006). "A common variant associated with prostate cancer in European and African populations." Nature genetics **38**(6): 652-658.
Andersson, H., A. M. D'Antona, D. A. Kendall, G. Von Heijne and C.-N. Chin (2003). "Membrane Assembly of the Cannabinoid Receptor 1: Impact of a Long N-Terminal Tail." Molecular Pharmacology **64**(3): 570-577.
Aragon-Ching, J. B. (2011). "Implications for chemoprevention of prostate cancer with intake for cruciferous vegetables." Asian Journal of Andrology **13**: 357-358.
Ashok, R. V. (2014). "Tumour Suppressor Mechanisms in the Control of Chromosome Stability: Insights from BRCA2." Mol. Cells **37**(2): 95-99.
Baca, S. C., D. Prandi, M. S. Lawrence, J. M. Mosquera, A. Romanel, Y. Drier, K. Park, N. Kitabayashi, T. Y. MacDonald, M. Ghandi, E. Van Allen, G. V. Kryukov, A. Sboner, J.-P. Theurillat, T. D. Soong, E. Nickerson, D. Auclair, A. Tewari, H. Beltran, R. C. Onofrio, G. Boysen, C. Guiducci, C. E. Barbieri, K. Cibulskis, A. Sivachenko, S. L. Carter, G. Saksena, D. Voet, A. H. Ramos, W. Winckler, M. Cipicchio, K. Ardlie, P. W. Kantoff, M. F. Berger, S. B. Gabriel, T. R. Golub, M. Meyerson, E. S. Lander, O. Elemento, G. Getz, F. Demichelis, M. A. Rubin and L. A. Garraway (2013). "Punctuated Evolution of Prostate Cancer Genomes." Cell **153**(3): 666-677.
Barbieri, C. E., S. C. Baca, M. S. Lawrence, F. Demichelis, M. Blattner, J.-P. Theurillat, T. A. White, P. Stojanov, E. Van Allen, N. Stransky, E. Nickerson, S.-S. Chae, G. Boysen, D. Auclair, R. C. Onofrio, K. Park, N. Kitabayashi, T. Y. MacDonald, K. Sheikh, T. Vuong, C. Guiducci, K. Cibulskis, A. Sivachenko, S. L. Carter, G. Saksena, D. Voet, W. M. Hussain, A. H. Ramos, W. Winckler, M. C. Redman, K. Ardlie, A. K. Tewari, J. M. Mosquera, N. Rupp, P. J. Wild, H. Moch, C. Morrissey, P. S. Nelson, P. W. Kantoff, S. B. Gabriel, T. R. Golub, M. Meyerson, E. S. Lander, G. Getz, M. A. Rubin and L. A. Garraway (2012). "Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer." Nature genetics **44**(6): 685-689.
Barbieri, C. E., C. H. Bangma, A. Bjartell, J. W. Catto, Z. Culig, H. Gronberg, J. Luo, T. Visakorpi and M. A. Rubin (2013). "The Mutational Landscape of Prostate Cancer." European urology **64**(4): 567-576.
Barbieri, C. E., C. H. Bangma, A. Bjartell, J. W. F. Catto, Z. Culig, H. Gr√ ∂ nberg, J. Luo, T. Visakorpi and M. A. Rubin (2013). "The Mutational Landscape of Prostate Cancer." European urology **64**(4): 567-576.
Basavarajappa, B. S., R. Yalamanchili, T. B. Cooper and B. L. Hungund (2008). The Endocannabinoid System. Handbook of Neurochemistry and Molecular Neurobiology. A. Lajtha and E. S. Vizi, Springer US**:** 343-384.

Bass, J. J., D. J. Wilkinson, D. Rankin, B. E. Phillips, N. J. Szewczyk, K. Smith and P. J. Atherton (2016). "An overview of technical considerations for Western blotting applications to physiological research." Scandinavian Journal of Medicine & Science in Sports: n/a-n/a.
Bedoya, F., J. C. Meneu, M. I. Macias, A. Moreno, R. Enriquez-de-Salamanca, E. M. Gonzalez and I. Vegh (2009). "Mutation in CNR1 gene and VEGF expression in esophageal cancer." Tumori **95**(1): 68-75.
Bedoya, F., J. C. Rubio, C. Morales-Gutierrez, A. Abad-Barahona, D. Lora Pablos, J. C. Meneu, E. Moreno-Gonzalez, R. Enriquez de Salamanca and I. Vegh (2009). "Single Nucleotide Change in the Cannabinoid Receptor-1 (CNR1) Gene in Colorectal Cancer Outcome." Oncology **76**(6): 435-441.
Beer, T. M., A. J. Armstrong, D. E. Rathkopf, Y. Loriot, C. N. Sternberg, C. S. Higano, P. Iversen, S. Bhattacharya, J. Carles, S. Chowdhury, I. D. Davis, J. S. de Bono, C. P. Evans, K. Fizazi, A. M. Joshua, C. S. Kim, G. Kimura, P. Mainwaring, H. Mansbach, K. Miller, S. B. Noonberg, F. Perabo, D. Phung, F. Saad, H. I. Scher, M. E. Taplin, P. M. Venner, B. Tombal and P. I. for the (2014). "Enzalutamide in Metastatic Prostate Cancer before Chemotherapy." The New England journal of medicine **371**(5): 424-433.
Beltran, H., R. Yelensky, G. M. Frampton, K. Park, S. R. Downing, T. Y. MacDonald, M. Jarosz, D. Lipson, S. T. Tagawa, D. M. Nanus, P. J. Stephens, J. M. Mosquera, M. T. Cronin and M. A. Rubin (2013). "Targeted Next-generation Sequencing of Advanced Prostate Cancer Identifies Potential Therapeutic Targets and Disease Heterogeneity." European urology **63**(5): 920-926.
Ben Liu, Q. M., Min Cao, and Liping Xie (2011). "Cruiferous vegetables intake and risk of prostate cancer: A meta-analysis." International Journal of Urology **19**: 134-141.
Berger, M. F., M. S. Lawrence, F. Demichelis, Y. Drier, K. Cibulskis, A. Y. Sivachenko, A. Sboner, R. Esgueva, D. Pflueger, C. Sougnez, R. Onofrio, S. L. Carter, K. Park, L. Habegger, L. Ambrogio, T. Fennell, M. Parkin, G. Saksena, D. Voet, A. H. Ramos, T. J. Pugh, J. Wilkinson, S. Fisher, W. Winckler, S. Mahan, K. Ardlie, J. Baldwin, J. W. Simons, N. Kitabayashi, T. Y. MacDonald, P. W. Kantoff, L. Chin, S. B. Gabriel, M. B. Gerstein, T. R. Golub, M. Meyerson, A. Tewari, E. S. Lander, G. Getz, M. A. Rubin and L. A. Garraway (2011). "The genomic complexity of primary human prostate cancer." Nature **470**(7333): 214-220.
Berndt, S. I., Z. Wang, M. Yeager, M. C. Alavanja, D. Albanes, L. Amundadottir, G. Andriole, L. Beane Freeman, D. Campa, G. Cancel-Tassin, F. Canzian, J.-N. Cornu, O. Cussenot, W. R. Diver, S. M. Gapstur, H. Gronberg, C. A. Haiman, B. Henderson, A. Hutchinson, D. J. Hunter, T. J. Key, S. Kolb, S. Koutros, P. Kraft, L. Le Marchand, S. Lindstrom, M. J. Machiela, E. A. Ostrander, E. Riboli, F. Schumacher, A. Siddiq, J. L. Stanford, V. L. Stevens, R. C. Travis, K. K. Tsilidis, J. Virtamo, S. Weinstein, F. Wilkund, J. Xu, S. Lilly Zheng, K. Yu, W. Wheeler, H. Zhang, G. C. African Ancestry Prostate Cancer, J. Sampson, A. Black, K. Jacobs, R. N. Hoover, M. Tucker and S. J. Chanock (2015). "Two susceptibility loci identified for prostate cancer aggressiveness." Nat Commun **6**.
Bethel, C. R., D. Faith, X. Li, B. Guan, J. L. Hicks, F. Lan, R. B. Jenkins, C. J. Bieberich and A. M. De Marzo (2006). "Decreased NKX3.1 Protein Expression in Focal Prostatic Atrophy, Prostatic Intraepithelial Neoplasia, and Adenocarcinoma: Association with Gleason Score and Chromosome 8p Deletion." Cancer Research **66**(22): 10683-10690.

Bifulco, M., C. Laezza, S. Pisanti and P. Gazzerro (2006). "Cannabinoids and cancer: pros and cons of an antitumour strategy." British Journal of Pharmacology **148**(2): 123-135.
Bingbing Wei, Z. X., Jun Ruan, Ming Zhu and Ke Jin, et al. (2012). "RNASEL Asp541Glu and Arg462Gln polymorphisms in prostate cancer risk: evidences from a meta-analysis

."

Molecular biology reports **39**(3): 2347-2353.
Bishop, J. (1987). "The molecular genetics of cancer." Science **235**(4786): 305-311.
Bishop, K. S., D. Y. Han, N. Karunasinghe, M. Goudie, J. G. Masters and L. R. Ferguson (2016). "An examination of clinical differences between carriers and non-carriers of chromosome 8q24 risk alleles in a New Zealand Caucasian population with prostate cancer." PeerJ **4**: e1731.
Boström, P. J., A. S. Bjartell, J. W. F. Catto, S. E. Eggener, H. Lilja, S. Loeb, J. Schalken, T. Schlomm and M. R. Cooperberg (2015). "Genomic Predictors of Outcome in Prostate Cancer." European Urology **68**(6): 1033-1044.
Boyd, L. K., X. Mao and Y.-J. Lu (2012). "The complexity of prostate cancer: genomic alterations and heterogeneity." Nat Rev Urol **9**(11): 652-664.
Boyd, L. K., X. Mao, L. Xue, D. Lin, T. Chaplin, S. C. Kudahetti, E. Stankiewicz, Y. Yu, L. Beltran, G. Shaw, J. Hines, R. T. D. Oliver, D. M. Berney, B. D. Young and Y.-J. Lu (2012). "High-resolution genome-wide copy-number analysis suggests a monoclonal origin of multifocal prostate cancer." Genes, Chromosomes and Cancer **51**(6): 579-589.
Boyle, P., A. Koechlin, M. Bota, A. d'Onofrio, D. G. Zaridze, P. Perrin, J. Fitzpatrick, A. L. Burnett and M. Boniol (2016). "Endogenous and exogenous testosterone and the risk of prostate cancer and increased prostate-specific antigen (PSA) level: a meta-analysis." BJU international: n/a-n/a.
Brown, I., M. G. Cascio, K. W. J. Wahle, R. Smoum, R. Mechoulam, R. A. Ross, R. G. Pertwee and S. D. Heys (2010). "Cannabinoid receptor-dependent and -independent anti-proliferative effects of omega-3 ethanolamides in androgen receptor-positive and -negative prostate cancer cell lines." Carcinogenesis **31**(9): 1584-1591.
Carmen Jero´nimo, P. J. B., Anders Bjartell, Giuseppina M. Carbone,James W.F. Catto, Susan J. Clark, Rui Henrique, William G. Nelson, Shahrokh F. Shariat (2011). "Epigenetics in Prostate Cancer: Biologic and Clinical Relevance." Eur Urol. **60**: 753-766.
Carracedo, A., M. Gironella, M. Lorente, S. Garcia, M. Guzm√° n, G. Velasco and J. L. Iovanna (2006). "Cannabinoids Induce Apoptosis of Pancreatic Tumor Cells via Endoplasmic Reticulum Stress, ÄìRelated Genes." Cancer research **66**(13): 6748-6755.
Carracedo, A., M. Gironella, M. Lorente, S. Garcia, M. Guzman, G. Velasco and J. L. Iovanna (2006). "Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes." Cancer research **66**(13): 6748-6755.

Carter, B. S., T. H. Beaty, G. D. Steinberg, B. Childs and P. C. Walsh (1992). "Mendelian inheritance of familial prostate cancer." Proceedings of the National Academy of Sciences of the United States of America **89**(8): 3367-3371.
Castillo, Pablo E., Thomas J. Younts, Andrés E. Chávez and Y. Hashimotodani (2012). "Endocannabinoid Signaling and Synaptic Function." Neuron **76**(1): 70-81.
Chakravarti, B., J. Ravi and R. K. Ganju (2014). "Cannabinoids as therapeutic agents in cancer: current status and future implications." Oncotarget **5**(15): 5852-5872.
Chakravarti, B., J. Ravi and R. K. Ganju (2014). Cannabinoids as therapeutic agents in cancer: current status and future implications.
Chen, C., H. V. Bhalala, R. L. Vessella and J. T. Dong (2003). "KLF5 is frequently deleted and down-regulated but rarely mutated in prostate cancer." The Prostate **55**(2): 81-88.
Chen, R., S. Ren, M. K. Yiu, N. C. Fai, W. S. Cheng, L. H. Ian, S. Naito, T. Matsuda, E. Kehinde, A. Kural, J. Y. Chiu, R. Umbas, Q. Wei, X. Shi, L. Zhou, J. Huang, Y. Huang, L. Xie, L. Ma, C. Yin, D. Xu, K. Xu, Z. Ye, C. Liu, D. Ye, X. Gao, Q. Fu, J. Hou, J. Yuan, D. He, T. Pan, Q. Ding, F. Jin, B. Shi, G. Wang, X. Liu, D. Wang, Z. Shen, X. Kong, W. Xu, Y. Deng, H. Xia, A. N. Cohen, X. Gao, C. Xu and Y. Sun (2014). "Prostate cancer in Asia: A collaborative report." Asian Journal of Urology **1**(1): 15-29.
Cheng, H. H., C. C. Pritchard, T. Boyd, P. S. Nelson and B. Montgomery (2015). "Biallelic Inactivation of BRCA2 in Platinum-sensitive Metastatic Castration-resistant Prostate Cancer." European urology.
Choi, S. Y., H. J. Kim, H. S. Cheong and S. C. Myung (2015). "The association of 5-alpha reductase type 2 (SRD5A2) gene polymorphisms with prostate cancer in a Korean population." Korean J Urol **56**(1): 19-30.
Chopra, S. and P. Rashid (2015). "Management of castration-resistant (advanced) prostate cancer (CRPC): rationale, progress and future directions." Australian Family Physician **44**: 302-305.
Christopher John Fowler, S. C. C. a. E. P. (2009). "Cannabinoid receptor-independent actions of HU210 upon the viability and mobility of PC3 prostate cancer cells." FASEB J.
Chung, S. C., P. Hammarsten, A. Josefsson, P. Stattin, T. Granfors, L. Egevad, G. Mancini, B. Lutz, A. Bergh and C. J. Fowler (2009). "A high cannabinoid CB1 receptor immunoreactivity is associated with disease severity and outcome in prostate cancer." European journal of cancer **45**(1): 174-182.
Cooper, C. S., R. Eeles, D. C. Wedge, P. Van Loo, G. Gundem, L. B. Alexandrov, B. Kremeyer, A. Butler, A. G. Lynch, N. Camacho, C. E. Massie, J. Kay, H. J. Luxton, S. Edwards, Z. Kote-Jarai, N. Dennis, S. Merson, D. Leongamornlert, J. Zamora, C. Corbishley, S. Thomas, S. Nik-Zainal, S. O'Meara, L. Matthews, J. Clark, R. Hurst, R. Mithen, R. G. Bristow, P. C. Boutros, M. Fraser, S. Cooke, K. Raine, D. Jones, A. Menzies, L. Stebbings, J. Hinton, J. Teague, S. McLaren, L. Mudie, C. Hardy, E. Anderson, O. Joseph, V. Goody, B. Robinson, M. Maddison, S. Gamble, C. Greenman, D. Berney, S. Hazell, N. Livni, I. P. G. The, C. Fisher, C. Ogden, P. Kumar, A. Thompson, C. Woodhouse, D. Nicol, E. Mayer, T. Dudderidge, N. C. Shah, V. Gnanapragasam, T. Voet, P. Campbell, A. Futreal, D. Easton, A. Y. Warren, C. S. Foster, M. R. Stratton, H. C. Whitaker, U. McDermott, D. S. Brewer and D. E. Neal (2015). "Analysis of the Genetic Phylogeny of Multifocal Prostate Cancer

Identifies Multiple Independent Clonal Expansions in Neoplastic and Morphologically Normal Prostate Tissue." Nature genetics **47**(4): 367-372.
Czifra, G., A. Varga, K. Nyeste, R. Marincsak, B. I. Toth, I. Kovacs, L. Kovacs and T. Biro (2009). "Increased expressions of cannabinoid receptor-1 and transient receptor potential vanilloid-1 in human prostate carcinoma." Journal of cancer research and clinical oncology **135**(4): 507-514.
Damber, J.-E. and G. Aus (2008). "Prostate cancer." the Lancet **371**(9625): 1710-1721.
de Bono, J. S., C. J. Logothetis, A. Molina, K. Fizazi, S. North, L. Chu, K. N. Chi, R. J. Jones, O. B. Goodman, F. Saad, J. N. Staffurth, P. Mainwaring, S. Harland, T. W. Flaig, T. E. Hutson, T. Cheng, H. Patterson, J. D. Hainsworth, C. J. Ryan, C. N. Sternberg, S. L. Ellard, A. Fléhon, M. Saleh, M. Scholz, E. Efstathiou, A. Zivi, D. Bianchini, Y. Loriot, N. Chieffo, T. Kheoh, C. M. Haqq, H. I. Scher and C. O. U. A. A. I. for the (2011). "Abiraterone and Increased Survival in Metastatic Prostate Cancer." The New England journal of medicine **364**(21): 1995-2005.
de Bono, J. S., S. Oudard, M. Ozguroglu, S. r. Hansen, J.-P. Machiels, I. Kocak, G. l. Gravis, I. Bodrogi, M. J. Mackenzie, L. Shen, M. Roessner, S. Gupta and A. O. Sartor (2010). "Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial." the Lancet **376**(9747): 1147-1154.
de Muga, S., S. Hernandez, L. Agell, M. Salido, N. Juanpere, M. Lorenzo, J. A. Lorente, S. Serrano and J. Lloreta (2010). "Molecular alterations of EGFR and PTEN in prostate cancer: association with high-grade and advanced-stage carcinomas." Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc **23**(5): 703-712.
De Petrocellis, L., D. Melck, A. Palmisano, T. Bisogno, C. Laezza, M. Bifulco and V. Di Marzo (1998). "The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation." Proceedings of the National Academy of Sciences **95**(14): 8375-8380.
Decker, B., D. M. Karyadi, B. W. Davis, E. Karlins, L. S. Tillmans, J. L. Stanford, S. N. Thibodeau and E. A. Ostrander (2016). "Biallelic BRCA2 Mutations Shape the Somatic Mutational Landscape of Aggressive Prostate Tumors." The American Journal of Human Genetics.
Delpu, Y., P. Cordelier, W. C. Cho and J. r. m. Torrisani (2013). "DNA Methylation and Cancer Diagnosis." International Journal of Molecular Sciences **14**(7): 15029-15058.
DeMarzo, A. M., W. G. Nelson, W. B. Isaacs and J. I. Epstein (2003). "Pathological and molecular aspects of prostate cancer." Lancet **361**(9361): 955-964.
Demuth, D. G. and A. Molleman (2006). "Cannabinoid signalling." Life sciences **78**(6): 549-563.
Demuth, D. G. and A. Molleman (2006). "Cannabinoid signalling." Life sciences **78**(6): 549-563.
Devane, W., L. Hanus, A. Breuer, R. Pertwee, L. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger and R. Mechoulam (1992). "Isolation and structure of a brain constituent that binds to the cannabinoid receptor." Science **258**(5090): 1946-1949.
Devane, W. A., F. A. Dysarz, M. R. Johnson, L. S. Melvin and A. C. Howlett (1988). "Determination and characterization of a cannabinoid receptor in rat brain." Molecular Pharmacology **34**(5): 605-613.

Devilee, P., A.-M. Cleton-Jansen and C. J. Cornelisse (2001). "Ever since Knudson." Trends in Genetics **17**(10): 569-573.
Di Marzo, V., D. Melck, T. Bisogno and L. De Petrocellis "Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action." Trends in Neurosciences **21**(12): 521-528.
Díaz-Laviada, I. (2011). "Tne endocannabinoid system in prostate cancer." Nature Reviews. Urology **8**: 553-561.
Dong, J. T. (2001). "Chromosomal deletions and tumor suppressor genes in prostate cancer." Cancer metastasis reviews **20**(3-4): 173-193.
Dong, J. T. (2006). "Prevalent mutations in prostate cancer." Journal of cellular biochemistry **97**(3): 433-447.
Dong, X., L. Wang, K. Taniguchi, X. Wang, J. M. Cunningham, S. K. McDonnell, C. Qian, A. F. Marks, S. L. Slager, B. J. Peterson, D. I. Smith, J. C. Cheville, M. L. Blute, S. J. Jacobsen, D. J. Schaid, D. J. Tindall, S. N. Thibodeau and W. Liu (2003). "Mutations in CHEK2 associated with prostate cancer risk." American journal of human genetics **72**(2): 270-280.
Dong, X.-Y., C. Rodriguez, P. Guo, X. Sun, J. T. Talbot, W. Zhou, J. Petros, Q. Li, R. L. Vessella, A. S. Kibel, V. L. Stevens, E. E. Calle and J.-T. Dong (2008). "SnoRNA U50 is a candidate tumor suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer." Human molecular genetics **17**(7): 1031-1042.
Dong, X. Y., C. Rodriguez, P. Guo, X. Sun, J. T. Talbot, W. Zhou, J. Petros, Q. Li, R. L. Vessella, A. S. Kibel, V. L. Stevens, E. E. Calle and J. T. Dong (2008). "SnoRNA U50 is a candidate tumor-suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer." Human molecular genetics **17**(7): 1031-1042.
Eckardt, K., H. Sell, A. Taube, M. Koenen, B. Platzbecker, A. Cramer, A. Horrighs, M. Lehtonen, N. Tennagels and J. Eckel (2009). "Cannabinoid type 1 receptors in human skeletal muscle cells participate in the negative crosstalk between fat and muscle." Diabetologia **52**(4): 664-674.
Eeles, R. A., Z. Kote-Jarai, A. A. Al Olama, G. G. Giles, M. Guy, G. Severi, K. Muir, J. L. Hopper, B. E. Henderson, C. A. Haiman, J. Schleutker, F. C. Hamdy, D. E. Neal, J. L. Donovan, J. L. Stanford, E. A. Ostrander, S. A. Ingles, E. M. John, S. N. Thibodeau, D. Schaid, J. Y. Park, A. Spurdle, J. Clements, J. L. Dickinson, C. Maier, W. Vogel, T. D√ ∂ rk, T. R. Rebbeck, K. A. Cooney, L. Cannon-Albright, P. O. Chappuis, P. Hutter, M. Zeegers, R. Kaneva, H. W. Zhang, Y. J. Lu, W. D. Foulkes, D. R. English, D. A. Leongamornlert, M. Tymrakiewicz, J. Morrison, A. T. Ardern-Jones, A. L. Hall, L. T. O'Brien, R. A. Wilkinson, E. J. Saunders, E. C. Page, E. J. Sawyer, S. M. Edwards, D. P. Dearnaley, A. Horwich, R. A. Huddart, V. S. Khoo, C. C. Parker, N. Van As, C. J. Woodhouse, A. Thompson, T. Christmas, C. Ogden, C. S. Cooper, M. C. Southey, A. Lophatananon, J. F. Liu, L. N. Kolonel, L. Le Marchand, T. Wahlfors, T. L. Tammela, A. Auvinen, S. J. Lewis, A. Cox, L. M. Fitzgerald, J. S. Koopmeiners, D. M. Karyadi, E. M. Kwon, M. C. Stern, R. Corral, A. D. Joshi, A. Shahabi, S. K. McDonnell, T. A. Sellers, J. Pow-Sang, S. Chambers, J. Aitken, R. A. Gardiner, J. Batra, M. A. Kedda, F. Lose, A. Polanowski, B. Patterson, J. Serth, A. Meyer, M. Luedeke, K. Stefflova, A. M. Ray, E. M. Lange, J. Farnham, H. Khan, C. Slavov, A. Mitkova, G. Cao and D. F. Easton (2009). "Identification of seven new prostate cancer susceptibility loci through a genome-wide association study." Nature genetics **41**(10): 1116-1121.

Eeles, R. A., Z. Kote-Jarai, A. A. Al Olama, G. G. Giles, M. Guy, G. Severi, K. Muir, J. L. Hopper, B. E. Henderson, C. A. Haiman, J. Schleutker, F. C. Hamdy, D. E. Neal, J. L. Donovan, J. L. Stanford, E. A. Ostrander, S. A. Ingles, E. M. John, S. N. Thibodeau, D. Schaid, J. Y. Park, A. Spurdle, J. Clements, J. L. Dickinson, C. Maier, W. Vogel, T. Dork, T. R. Rebbeck, K. A. Cooney, L. Cannon-Albright, P. O. Chappuis, P. Hutter, M. Zeegers, R. Kaneva, H.-W. Zhang, Y.-J. Lu, W. D. Foulkes, D. R. English, D. A. Leongamornlert, M. Tymrakiewicz, J. Morrison, A. T. Ardern-Jones, A. L. Hall, L. T. O'Brien, R. A. Wilkinson, E. J. Saunders, E. C. Page, E. J. Sawyer, S. M. Edwards, D. P. Dearnaley, A. Horwich, R. A. Huddart, V. S. Khoo, C. C. Parker, N. Van As, C. J. Woodhouse, A. Thompson, T. Christmas, C. Ogden, C. S. Cooper, M. C. Southey, A. Lophatananon, J.-F. Liu, L. N. Kolonel, L. Le Marchand, T. Wahlfors, T. L. Tammela, A. Auvinen, S. J. Lewis, A. Cox, L. M. FitzGerald, J. S. Koopmeiners, D. M. Karyadi, E. M. Kwon, M. C. Stern, R. Corral, A. D. Joshi, A. Shahabi, S. K. McDonnell, T. A. Sellers, J. Pow-Sang, S. Chambers, J. Aitken, R. A. Gardiner, J. Batra, M. A. Kedda, F. Lose, A. Polanowski, B. Patterson, J. Serth, A. Meyer, M. Luedeke, K. Stefflova, A. M. Ray, E. M. Lange, J. Farnham, H. Khan, C. Slavov, A. Mitkova, G. Cao and D. F. Easton (2009). "Identification of seven new prostate cancer susceptibility loci through a genome-wide association study." Nature genetics **41**(10): 1116-1121.

Eeles, R. A., A. A. A. Olama, S. Benlloch, E. J. Saunders, D. A. Leongamornlert, M. Tymrakiewicz, M. Ghoussaini, C. Luccarini, J. Dennis, S. Jugurnauth-Little, T. Dadaev, D. E. Neal, F. C. Hamdy, J. L. Donovan, K. Muir, G. G. Giles, G. Severi, F. Wiklund, H. Gronberg, C. A. Haiman, F. Schumacher, B. E. Henderson, L. Le Marchand, S. Lindstrom, P. Kraft, D. J. Hunter, S. Gapstur, S. J. Chanock, S. I. Berndt, D. Albanes, G. Andriole, J. Schleutker, M. Weischer, F. Canzian, E. Riboli, T. J. Key, R. C. Travis, D. Campa, S. A. Ingles, E. M. John, R. B. Hayes, P. D. P. Pharoah, N. Pashayan, K.-T. Khaw, J. L. Stanford, E. A. Ostrander, L. B. Signorello, S. N. Thibodeau, D. Schaid, C. Maier, W. Vogel, A. S. Kibel, C. Cybulski, J. Lubinski, L. Cannon-Albright, H. Brenner, J. Y. Park, R. Kaneva, J. Batra, A. B. Spurdle, J. A. Clements, M. R. Teixeira, E. Dicks, A. Lee, A. M. Dunning, C. Baynes, D. Conroy, M. J. Maranian, S. Ahmed, K. Govindasami, M. Guy, R. A. Wilkinson, E. J. Sawyer, A. Morgan, D. P. Dearnaley, A. Horwich, R. A. Huddart, V. S. Khoo, C. C. Parker, N. J. Van As, C. J. Woodhouse, A. Thompson, T. Dudderidge, C. Ogden, C. S. Cooper, A. Lophatananon, A. Cox, M. C. Southey, J. L. Hopper, D. R. English, M. Aly, J. Adolfsson, J. Xu, S. L. Zheng, M. Yeager, R. Kaaks, W. R. Diver, M. M. Gaudet, M. C. Stern, R. Corral, A. D. Joshi, A. Shahabi, T. Wahlfors, T. L. J. Tammela, A. Auvinen, J. Virtamo, P. Klarskov, B. G. Nordestgaard, M. A. Roder, S. F. Nielsen, S. E. Bojesen, A. Siddiq, L. M. FitzGerald, S. Kolb, E. M. Kwon, D. M. Karyadi, W. J. Blot, W. Zheng, Q. Cai, S. K. McDonnell, A. E. Rinckleb, B. Drake, G. Colditz, D. Wokolorczyk, R. A. Stephenson, C. Teerlink, H. Muller, D. Rothenbacher, T. A. Sellers, H.-Y. Lin, C. Slavov, V. Mitev, F. Lose, S. Srinivasan, S. Maia, P. Paulo, E. Lange, K. A. Cooney, A. C. Antoniou, D. Vincent, F. Bacot, D. C. Tessier, Z. Kote-Jarai and D. F. Easton (2013). "Identification of 23 new prostate cancer susceptibility loci using the iCOGS custom genotyping array." Nature genetics **45**(4): 385-391.

Egertova, M. and M. R. Elphick (2000). "Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB." The Journal of comparative neurology **422**(2): 159-171.

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl (2001). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-498.

Ellert-Miklaszewska, A., I. Ciechomska and B. Kaminska (2013). Cannabinoid Signaling in Glioma Cells. Glioma Signaling. J. Bara≈Ñska, Springer Netherlands. **986:** 209-220.
Ewing, C. M., A. M. Ray, E. M. Lange, K. A. Zuhlke, C. M. Robbins, W. D. Tembe, K. E. Wiley, S. D. Isaacs, D. Johng, Y. Wang, C. Bizon, G. Yan, M. Gielzak, A. W. Partin, V. Shanmugam, T. Izatt, S. Sinari, D. W. Craig, S. L. Zheng, P. C. Walsh, J. E. Montie, J. Xu, J. D. Carpten, W. B. Isaacs and K. A. Cooney (2012). "Germline Mutations in HOXB13 and Prostate-Cancer Risk." New England Journal of Medicine **366**(2): 141-149.
Fang, Z. and X. Cui (2011). "Design and validation issues in RNA-seq experiments." Briefings in Bioinformatics **12**(3): 280-287.
FitzGerald, L. M., A. Kumar, E. A. Boyle, Y. Zhang, L. M. McIntosh, S. Kolb, M. Stott-Miller, T. Smith, D. M. Karyadi, E. A. Ostrander, L. Hsu, J. Shendure and J. L. Stanford (2013). "Germline Missense Variants in the BTNL2 Gene Are Associated with Prostate Cancer Susceptibility." Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology **22**(9): 1520-1528.
Fowler, C. J. (2013). "Transport of endocannabinoids across the plasma membrane and within the cell." The FEBS journal **280**(9): 1895-1904.
Fowler, C. J., A. Josefsson, L. Thors, S. C. Chung, P. Hammarsten, P. Wikstrom and A. Bergh (2013). "Tumour epithelial expression levels of endocannabinoid markers modulate the value of endoglin-positive vascular density as a prognostic marker in prostate cancer." Biochimica et biophysica acta **1831**(10): 1579-1587.
Frank, S. B. and C. K. Miranti (2013). "Disruption of prostate epithelial differentiation pathways and prostate cancer development." Frontiers in Oncology **3**.
Freimuth, N., R. Ramer and B. Hinz (2010). "Antitumorigenic effects of cannabinoids beyond apoptosis." Journal of Pharmacology and Experimental Therapeutics **332**(2): 336-344.
Galve-Roperh, I., C. Sanchez, M. L. Cortes, T. G. del Pulgar, M. Izquierdo and M. Guzman (2000). "Anti-tumoral action of cannabinoids: Involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation." Nat Med **6**(3): 313-319.
Gao, D., I. Vela, A. Sboner, P. J. Iaquinta, W. R. Karthaus, A. Gopalan, C. Dowling, J. N. Wanjala, E. A. Undvall, V. K. Arora, J. Wongvipat, M. Kossai, S. Ramazanoglu, L. P. Barboza, W. Di, Z. Cao, Q. F. Zhang, I. Sirota, L. Ran, T. Y. MacDonald, H. Beltran, J.-M. Mosquera, K. A. Touijer, P. T. Scardino, V. P. Laudone, K. R. Curtis, D. E. Rathkopf, M. J. Morris, D. C. Danila, S. F. Slovin, S. B. Solomon, J. A. Eastham, P. Chi, B. Carver, M. A. Rubin, H. I. Scher, H. Clevers, C. L. Sawyers and Y. Chen (2014). "Organoid Cultures Derived from Patients with Advanced Prostate Cancer." Cell **159**(1): 176-187.
Gaoni, Y. and R. Mechoulam (1964). "Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish." Journal of the American Chemical Society **86**(8): 1646-1647.
Gilgun-Sherki, Y., E. Melamed, R. Mechoulam and D. Offen (2003). "The CB1 Cannabinoid Receptor Agonist, HU-210, Reduces Levodopa-Induced Rotations in 6-Hydroxydopamine-Lesioned Rats." Pharmacology & Toxicology **93**(2): 66-70.

Gleason, D. F. and Mellinge.Gt (1974). "PREDICTION OF PROGNOSIS FOR PROSTATIC ADENOCARCINOMA BY COMBINED HISTOLOGICAL GRADING AND CLINICAL STAGING." Journal of Urology **111**(1): 58-64.
Gleason, D. F. and G. T. Mellinger (1974). "Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging." The Journal of urology **111**(1): 58-64.
Gleason, D. F. and G. T. Mellinger (2002). "Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. 1974." The Journal of urology **167**(2 Pt 2): 953-958; discussion 959.
Goldstein, A. S., J. Huang, C. Guo, I. P. Garraway and O. N. Witte (2010). "Identification of a cell-of-origin for human prostate cancer." Science (New York, N.Y.) **329**(5991): 568-571.
Gomella, L. G. (2009). "Effective Testosterone Suppression for Prostate Cancer: Is There a Best Castration Therapy?" Reviews in Urology **11**(2): 52-60.
Gong, M., W. Dong, Z. Shi, Y. Xu, W. Ni and R. An (2012). "Genetic Polymorphisms of GSTM1, GSTT1, and GSTP1 with Prostate Cancer Risk: A Meta-Analysis of 57 Studies." PloS one **7**(11): e50587.
Graff, J. N. and E. D. Chamberlain (2015). "Sipuleucel-T in the treatment of prostate cancer: an evidence-based review of its place in therapy." Core Evidence **10**: 1-10.
Grasso, C. S., Y.-M. Wu, D. R. Robinson, X. Cao, S. M. Dhanasekaran, A. P. Khan, M. J. Quist, X. Jing, R. J. Lonigro, J. C. Brenner, I. A. Asangani, B. Ateeq, S. Y. Chun, J. Siddiqui, L. Sam, M. Anstett, R. Mehra, J. R. Prensner, N. Palanisamy, G. A. Ryslik, F. Vandin, B. J. Raphael, L. P. Kunju, D. R. Rhodes, K. J. Pienta, A. M. Chinnaiyan and S. A. Tomlins (2012). "The Mutational Landscape of Lethal Castrate Resistant Prostate Cancer." Nature **487**(7406): 239-243.
Green, G. A., A. L. Hanlon, T. Al-Saleem and G. E. Hanks (1998). "A Gleason score of 7 predicts a worse outcome for prostate carcinoma patients treated with radiotherapy." Cancer **83**(5): 971-976.
Gregoire Robert, S., Frank Smit, Tilly Alders, et al (2012). "Pational basis for combination of PCA3 and TMPRSS2:ERG gene fusion for prostate cancer diagnosis." The prostate: 1-8.
Grimaldi, C., S. Pisanti, C. Laezza, A. M. Malfitano, A. Santoro, M. Vitale, M. G. Caruso, M. Notarnicola, I. Iacuzzo, G. Portella, V. Di Marzo and M. Bifulco (2006). "Anandamide inhibits adhesion and migration of breast cancer cells." Experimental Cell Research **312**(4): 363-373.
Grimsey, N. L., C. E. Goodfellow, E. L. Scotter, M. J. Dowie, M. Glass and E. S. Graham (2008). "Specific detection of CB1 receptors; cannabinoid CB1 receptor antibodies are not all created equal!" Journal of neuroscience methods **171**(1): 78-86.
Groskopf, J., S. M. J. Aubin, I. L. Deras, A. Blase, S. Bodrug, C. Clark, S. Brentano, J. Mathis, J. Pham, T. Meyer, M. Cass, P. Hodge, M. L. Macairan, L. S. Marks and H. Rittenhouse (2006). "APTIMA PCA3 molecular urine test: Development of a method to aid in the diagnosis of prostate cancer." Clinical Chemistry **52**(6): 1089-1095.
Gu, G. and A. R. Brothman (2011). "Cytogenomic aberrations associated with prostate cancer." Cancer genetics **204**(2): 57-67.

Gu, M., X. Dong, X. Zhang and W. Niu (2012). "The CAG repeat polymorphism of androgen receptor gene and prostate cancer: a meta-analysis." Molecular biology reports **39**(3): 2615-2624.
Gudmundsson, J., P. Sulem, A. Manolescu, L. T. Amundadottir, D. Gudbjartsson, A. Helgason, T. Rafnar, J. T. Bergthorsson, B. A. Agnarsson, A. Baker, A. Sigurdsson, K. R. Benediktsdottir, M. Jakobsdottir, J. Xu, T. Blondal, J. Kostic, J. Sun, S. Ghosh, S. N. Stacey, M. Mouy, J. Saemundsdottir, V. M. Backman, K. Kristjansson, A. Tres, A. W. Partin, M. T. Albers-Akkers, J. Godino-Ivan Marcos, P. C. Walsh, D. W. Swinkels, S. Navarrete, S. D. Isaacs, K. K. Aben, T. Graif, J. Cashy, M. Ruiz-Echarri, K. E. Wiley, B. K. Suarez, J. A. Witjes, M. Frigge, C. Ober, E. Jonsson, G. V. Einarsson, J. I. Mayordomo, L. A. Kiemeney, W. B. Isaacs, W. J. Catalona, R. B. Barkardottir, J. R. Gulcher, U. Thorsteinsdottir, A. Kong and K. Stefansson (2007). "Genome-wide association study identifies a second prostate cancer susceptibility variant at 8q24." Nature genetics **39**(5): 631-637.
Gudmundsson, J., P. Sulem, V. Steinthorsdottir, J. T. Bergthorsson, G. Thorleifsson, A. Manolescu, T. Rafnar, D. Gudbjartsson, B. A. Agnarsson, A. Baker, A. Sigurdsson, K. R. Benediktsdottir, M. Jakobsdottir, T. Blondal, S. N. Stacey, A. Helgason, S. Gunnarsdottir, A. Olafsdottir, K. T. Kristinsson, B. Birgisdottir, S. Ghosh, S. Thorlacius, D. Magnusdottir, G. Stefansdottir, K. Kristjansson, Y. Bagger, R. L. Wilensky, M. P. Reilly, A. D. Morris, C. H. Kimber, A. Adeyemo, Y. Chen, J. Zhou, W.-Y. So, P. C. Y. Tong, M. C. Y. Ng, T. Hansen, G. Andersen, K. Borch-Johnsen, T. Jorgensen, A. Tres, F. Fuertes, M. Ruiz-Echarri, L. Asin, B. Saez, E. van Boven, S. Klaver, D. W. Swinkels, K. K. Aben, T. Graif, J. Cashy, B. K. Suarez, O. van Vierssen Trip, M. L. Frigge, C. Ober, M. H. Hofker, C. Wijmenga, C. Christiansen, D. J. Rader, C. N. A. Palmer, C. Rotimi, J. C. N. Chan, O. Pedersen, G. Sigurdsson, R. Benediktsson, E. Jonsson, G. V. Einarsson, J. I. Mayordomo, W. J. Catalona, L. A. Kiemeney, R. B. Barkardottir, J. R. Gulcher, U. Thorsteinsdottir, A. Kong and K. Stefansson (2007). "Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes." Nature genetics **39**(8): 977-983.
Guindon, J. and A. G. Hohmann (2011). "The endocannabinoid system and cancer: therapeutic implication." British Journal of Pharmacology **163**(7): 1447-1463.
Gupta-Elera, G., A. R. Garrett, R. A. Robison and K. L. O,ÄôNeill (2012). "The role of oxidative stress in prostate cancer." European Journal of Cancer Prevention **21**(2): 155-162.
Gustafsson, S. B., R. Palmqvist, M. L. Henriksson, A. M. Dahlin, S. Edin, S. O. P. Jacobsson, Å. Öberg and C. J. Fowler (2011). "High Tumour Cannabinoid CB₁ Receptor Immunoreactivity Negatively Impacts Disease-Specific Survival in Stage II Microsatellite Stable Colorectal Cancer." PLoS ONE **6**(8): e23003.
Haiman, C. A., G. K. Chen, W. J. Blot, S. S. Strom, S. I. Berndt, R. A. Kittles, B. A. Rybicki, W. B. Isaacs, S. A. Ingles, J. L. Stanford, W. R. Diver, J. S. Witte, A. W. Hsing, B. Nemesure, T. R. Rebbeck, K. A. Cooney, J. Xu, A. S. Kibel, J. J. Hu, E. M. John, S. M. Gueye, S. Watya, L. B. Signorello, R. B. Hayes, Z. Wang, E. Yeboah, Y. Tettey, Q. Cai, S. Kolb, E. A. Ostrander, C. Zeigler-Johnson, Y. Yamamura, C. Neslund-Dudas, J. Haslag-Minoff, W. Wu, V. Thomas, G. O. Allen, A. Murphy, B.-L. Chang, S. L. Zheng, M. C. Leske, S.-Y. Wu, A. M. Ray, A. J. M. Hennis, M. J. Thun, J. Carpten, G. Casey, E. N. Carter, E. R. Duarte, L. Y. Xia, X. Sheng, P. Wan, L. C. Pooler, I. Cheng, K. R. Monroe, F. Schumacher, L. Le Marchand, L. N. Kolonel, S. J. Chanock, D. V. D. Berg,

D. O. Stram and B. E. Henderson (2011). "Genome-wide association study of prostate cancer in men of African ancestry identifies a susceptibility locus at 17q21." Nature genetics **43**(6): 570-573.
Haiman, C. A., N. Patterson, M. L. Freedman, S. R. Myers, M. C. Pike, A. Waliszewska, J. Neubauer, A. Tandon, C. Schirmer, G. J. McDonald, S. C. Greenway, D. O. Stram, L. L. Marchand, L. N. Kolonel, M. Frasco, D. Wong, L. C. Pooler, K. Ardlie, I. Oakley-Girvan, A. S. Whittemore, K. A. Cooney, E. M. John, S. A. Ingles, D. Altshuler, B. E. Henderson and D. Reich (2007). "Multiple regions within 8q24 independently affect risk for prostate cancer." Nature genetics **39**(5): 638-644.
Han, L., K. C. Vickers, D. C. Samuels and Y. Guo (2015). "Alternative applications for distinct RNA sequencing strategies." Briefings in Bioinformatics **16**(4): 629-639.
Han, Y., K. A. Rand, D. J. Hazelett, S. A. Ingles, R. A. Kittles, S. S. Strom, B. A. Rybicki, B. Nemesure, W. B. Isaacs, J. L. Stanford, W. Zheng, F. R. Schumacher, S. I. Berndt, Z. Wang, J. Xu, N. Rohland, D. Reich, A. Tandon, B. Pasaniuc, A. Allen, D. Quinque, S. Mallick, D. Notani, M. G. Rosenfeld, R. S. Jayani, S. Kolb, S. M. Gapstur, V. L. Stevens, C. A. Pettaway, E. D. Yeboah, Y. Tettey, R. B. Biritwum, A. A. Adjei, E. Tay, A. Truelove, S. Niwa, A. P. Chokkalingam, E. M. John, A. B. Murphy, L. B. Signorello, J. Carpten, M. C. Leske, S.-Y. Wu, A. J. M. Hennis, C. Neslund-Dudas, A. W. Hsing, L. Chu, P. J. Goodman, E. A. Klein, S. L. Zheng, J. S. Witte, G. Casey, A. Lubwama, L. C. Pooler, X. Sheng, G. A. Coetzee, M. B. Cook, S. J. Chanock, D. O. Stram, S. Watya, W. J. Blot, D. V. Conti, B. E. Henderson and C. A. Haiman (2016). "Prostate Cancer Susceptibility in Men of African Ancestry at 8q24." Journal of the National Cancer Institute **108**(7).
Hanahan, D. and Robert A. Weinberg "Hallmarks of Cancer: The Next Generation." Cell **144**(5): 646-674.
Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of Cancer: The Next Generation." Cell **144**(5): 646-674.
Hanus, L., S. Abu-Lafi, E. Fride, A. Breuer, Z. Vogel, D. E. Shalev, I. Kustanovich and R. Mechoulam (2001). "2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor." Proceedings of the National Academy of Sciences of the United States of America **98**(7): 3662-3665.
Heidenreich, A., G. Aus, M. Bolla, S. Joniau, V. B. Matveev, H. P. Schmid and F. Zattoni (2009). "[EAU guidelines on prostate cancer]." Actas urologicas espanolas **33**(2): 113-126.
Helfand, B. T., K. A. Roehl, P. R. Cooper, B. McGuire, L. M. Fitzgerald, G. Cancel-Tassin, J.-N. Cornu, S. Bauer, E. L. Van Blarigan, X. Chen, D. Duggan, E. A. Ostrander, M. Gwo-Shu, Z.-F. Zhang, S.-C. Chang, S. Jeong, S. Berndt, S. K. McDonnell, R. Kittles, B. A. Rybicki, M. Freedman, P. Kantoff, M. Pomerantz, J. P. Breyer, J. R. Smith, T. R. Rebbeck, D. Mercola, W. B. Isaacs, F. Wiklund, O. Cussenot, S. N. Thibodeau, D. J. Schaid, L. Cannon-Albright, K. A. Cooney, S. J. Chanock, J. L. Stanford, J. M. Chan, J. Witte, J. Xu, J. T. Bensen, J. A. Taylor and W. J. Catalona (2015). "Associations of Prostate Cancer Risk Variants with Disease Aggressiveness: Results of the NCI-SPORE Genetics Working Group Analysis of 18,343 Cases." Human genetics **134**(4): 439-450.
Hermanson, D. J. and L. J. Marnett (2011). "Cannabinoids, Endocannabinoids and Cancer." Cancer Metastasis Reviews **30**(3-4): 599-612.
Hermanson, D. J. and L. J. Marnett (2011). "Cannabinoids, endocannabinoids, and cancer." Cancer and Metastasis Reviews **30**(3-4): 599-612.

Hori, S., E. Butler and J. McLoughlin (2011). "Prostate cancer and diet: food for thought?" BJU international **107**(9): 1348-1359.
Hotte, S. J. and F. Saad (2010). "Current management of castrate-resistant prostate cancer." Current Oncology **17**(Suppl 2): S72-S79.
http://info.cancerresearchuk.org.
http://www.ensembl.org/Homo_sapiens/Gene/Sequence?g=ENSG00000118432;r=6:88849583-88876078.
https://www.caymanchem.com/Home.
Humphrey, P. A. (2004). "Gleason grading and prognostic factors in carcinoma of the prostate." Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc **17**(3): 292-306.
Huusko, P., D. Ponciano-Jackson, M. Wolf, J. A. Kiefer, D. O. Azorsa, S. Tuzmen, D. Weaver, C. Robbins, T. Moses, M. Allinen, S. Hautaniemi, Y. Chen, A. Elkahloun, M. Basik, G. S. Bova, L. Bubendorf, A. Lugli, G. Sauter, J. Schleutker, H. Ozcelik, S. Elowe, T. Pawson, J. M. Trent, J. D. Carpten, O. P. Kallioniemi and S. Mousses (2004). "Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer." Nature genetics **36**(9): 979-983.
Iacono, M. L., C. Buttigliero, V. Monica, E. Bollito, D. Garrou, S. Cappia, I. Rapa, F. Vignani, V. Bertaglia and C. Fiori (2016). "Retrospective study testing next generation sequencing of selected cancer-associated genes in resected prostate cancer." Oncotarget.
Idris, A. I. (2012). "The promise and dilemma of cannabinoid therapy: lessons from animal studies of bone disease." BoneKEy Rep **1**: 224.
James, N. D., M. R. Spears, N. W. Clarke, D. P. Dearnaley, J. S. De Bono, J. Gale, J. Hetherington, P. J. Hoskin, R. J. Jones, R. Laing, J. F. Lester, D. McLaren, C. C. Parker, M. K. B. Parmar, A. W. S. Ritchie, J. M. Russell, R. t. T. Strebel, G. N. Thalmann, M. D. Mason and M. R. Sydes (2015). "Survival with Newly Diagnosed Metastatic Prostate Cancer in the ‚ÄúDocetaxel Era‚Äù: Data from 917 Patients in the Control Arm of the STAMPEDE Trial (MRC PR08, CRUK/06/019)." European urology **67**(6): 1028-1038.
Javid, F. A., R. M. Phillips, S. Afshinjavid, R. Verde and A. Ligresti (2016). "Cannabinoid pharmacology in cancer research: A new hope for cancer patients?" European journal of pharmacology **775**: 1-14.
Jingwi, E. Y., M. Abbas, L. Ricks-Santi, D. Winchester, D. Beyene, A. Day, T. J. Naab, O. O. Kassim, G. M. Dunston and R. L. COPELAND (2015). "Vitamin D receptor genetic polymorphisms are associated with PSA level, Gleason score and prostate cancer risk in African-American men." Anticancer Research **35**(3): 1549-1558.
Joseph, J., B. Niggemann, K. S. Zaenker and F. Entschladen (2004). "Anandamide is an endogenous inhibitor for the migration of tumor cells and T lymphocytes." Cancer Immunology, Immunotherapy **53**(8): 723-728.
Joshi, A. D., R. n. Corral, C. Catsburg, J.-P. Lewinger, J. Koo, E. M. John, S. Ingles and M. C. Stern (2012). "Red meat and poultry, cooking practices, genetic susceptibility and risk of prostate cancer: results from the California Collaborative Prostate Cancer Study." Carcinogenesis.
Jung, C. K., W. K. Kang, J. M. Park, H. J. Ahn, S. W. Kim, S. Taek Oh and K. Y. Choi (2013). "Expression of the cannabinoid type I receptor and prognosis following surgery in colorectal cancer." Oncology Letters **5**(3): 870-876.
Kamiyama, M., M. Fukasawa, Y. Takihana, N. Sawada, H. Nakagomi, M. Yoshiyama, I. Araki and M. Takeda (2013). "Distribution and Possible Function of

Cannabinoid Receptor Subtype 1 in the Human Prostate." Open Journal of Urology **03**(02): 102-109.
Kantoff, P. W., C. S. Higano, N. D. Shore, E. R. Berger, E. J. Small, D. F. Penson, C. H. Redfern, A. C. Ferrari, R. Dreicer and R. B. Sims (2010). "Sipuleucel-T immunotherapy for castration-resistant prostate cancer." New England Journal of Medicine **363**(5): 411-422.
Karishma S Amin, P. P. B. (2012). "The cellular functions of RASSF1A and its inactivation in prostate cancer." Journal of Carcinogenesis **11**(3): 31-38.
Katzenwadel, A. and P. Wolf (2015). "Androgen deprivation of prostate cancer: Leading to a therapeutic dead end." Cancer letters **367**(1): 12-17.
Kelly, G. M., Y. H. Kong, A. Dobi, S. Srivastava, I. A. Sesterhenn, R. Pathmanathan, H. M. Tan, S.-H. Tan and S. C. Cheong (2015). "ERG oncoprotein expression in prostate carcinoma patients of different ethnicities." Molecular and Clinical Oncology **3**(1): 23-30.
Key, T. (2011). "Fruit and vegetables and cancer risk: Minireview." British Journal of Cancer **104**: 6-11.
Kheirandish, P. and F. Chinegwundoh (2011). "Ethnic differences in prostate cancer." British journal of cancer **105**(4): 481-485.
Kim, J. and J. Yu (2012). "Interrogating genomic and epigenomic data to understand prostate cancer." Biochimica et Biophysica Acta (BBA) - Reviews on Cancer **1825**(2): 186-196.
Kirby, R. S. a. M. I. P. (2008). " Prostate cancer." Abrington: Health **Fifth Edition**: 120
Klap, J., M. Schmid and K. R. Loughlin (2015). "The Relationship between Total Testosterone Levels and Prostate Cancer: A Review of the Continuing Controversy." The Journal of urology **193**(2): 403-414.
Knudson, A. G. (2001). "Two genetic hits (more or less) to cancer." Nat Rev Cancer **1**(2): 157-162.
Koboldt, D. C., Q. Zhang, D. E. Larson, D. Shen, M. D. McLellan, L. Lin, C. A. Miller, E. R. Mardis, L. Ding and R. K. Wilson (2012). "VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing." Genome Res **22**(3): 568-576.
Kote-Jarai, Z., A. A. A. Olama, G. G. Giles, G. Severi, J. Schleutker, M. Weischer, D. Campa, E. Riboli, T. Key, H. Gronberg, D. J. Hunter, P. Kraft, M. J. Thun, S. Ingles, S. Chanock, D. Albanes, R. B. Hayes, D. E. Neal, F. C. Hamdy, J. L. Donovan, P. Pharoah, F. Schumacher, B. E. Henderson, J. L. Stanford, E. A. Ostrander, K. D. Sorensen, T. Dork, G. Andriole, J. L. Dickinson, C. Cybulski, J. Lubinski, A. Spurdle, J. A. Clements, S. Chambers, J. Aitken, R. A. F. Gardiner, S. N. Thibodeau, D. Schaid, E. M. John, C. Maier, W. Vogel, K. A. Cooney, J. Y. Park, L. Cannon-Albright, H. Brenner, T. Habuchi, H.-W. Zhang, Y.-J. Lu, R. Kaneva, K. Muir, S. Benlloch, D. A. Leongamornlert, E. J. Saunders, M. Tymrakiewicz, N. Mahmud, M. Guy, L. T. O'Brien, R. A. Wilkinson, A. L. Hall, E. J. Sawyer, T. Dadaev, J. Morrison, D. P. Dearnaley, A. Horwich, R. A. Huddart, V. S. Khoo, C. C. Parker, N. Van As, C. J. Woodhouse, A. Thompson, T. Christmas, C. Ogden, C. S. Cooper, A. Lophatonanon, M. C. Southey, J. L. Hopper, D. R. English, T. Wahlfors, T. L. J. Tammela, P. Klarskov, B. G. Nordestgaard, M. A. Roder, A. Tybjaerg-Hansen, S. E. Bojesen, R. Travis, F. Canzian, R. Kaaks, F. Wiklund, M. Aly, S. Lindstrom, W. R. Diver, S. Gapstur, M. C. Stern, R. Corral, J. Virtamo, A. Cox, C. A. Haiman, L. Le Marchand, L. FitzGerald, S. Kolb, E. M. Kwon, D. M. Karyadi, T. F. Orntoft, M. Borre, A. Meyer, J. Serth, M.

Yeager, S. I. Berndt, J. R. Marthick, B. Patterson, D. Wokolorczyk, J. Batra, F. Lose, S. K. McDonnell, A. D. Joshi, A. Shahabi, A. E. Rinckleb, A. Ray, T. A. Sellers, H.-Y. Lin, R. A. Stephenson, J. Farnham, H. Muller, D. Rothenbacher, N. Tsuchiya, S. Narita, G.-W. Cao, C. Slavov, V. Mitev, D. F. Easton and R. A. Eeles (2011). "Seven prostate cancer susceptibility loci identified by a multi-stage genome-wide association study." Nature genetics **43**(8): 785-791.
Kumar-Sinha, C., S. A. Tomlins and A. M. Chinnaiyan (2008). "Recurrent gene fusions in prostate cancer." Nat Rev Cancer **8**(7): 497-511.
Laezza, C., S. Pisanti, A. M. Malfitano and M. Bifulco (2008). "The anandamide analog, Met-F-AEA, controls human breast cancer cell migration via the RHOA/RHO kinase signaling pathway." Endocrine-Related Cancer **15**(4): 965-974.
Lane, T. M., J. C. Strefford, R. J. Y√° √±ez-Mu√±oz, P. Purkis, E. Forsythe, T. Nia, J. Hines, Y.-J. Lu and R. T. Oliver (2007). "Identification of a Recurrent t(4;6) Chromosomal Translocation in Prostate Cancer." The Journal of urology **177**(5): 1907-1912.
Langmead, B. and S. L. Salzberg (2012). "Fast gapped-read alignment with Bowtie 2." Nat Methods **9**(4): 357-359.
Laprairie, R. B., M. E. M. Kelly and E. M. Denovan-Wright (2012). "The dynamic nature of type 1 cannabinoid receptor (CB1) gene transcription." British Journal of Pharmacology **167**(8): 1583-1595.
Larrinaga, G., S. Begoaa, P. Itxaro, L. Blanco, L. C. Maria, F. M. Pinto, J. Gil and I. L. p. Jos√© (2010). "Cannabinoid CB1 Receptor Is Downregulated in Clear Cell Renal Cell Carcinoma." Journal of Histochemistry & Cytochemistry **58**(12): 1129-1134.
Lars Budaus, M. B., Alberto Bossi, et al (2012). "Functional outcomes and complications following radiation therapy for prostate cancer: A critical analysis of the literature." Eurpean Urology **61**: 112-127.
Lee, A. and S. J. Chia (2015). "Contemporary outcomes in the detection of¬†prostate cancer using transrectal ultrasound-guided 12-core biopsy in Singaporean men with elevated prostate specific antigen and/or abnormal¬†digital rectal examination." Asian Journal of Urology **2**(4): 187-193.
Li, J., C. Yen, D. Liaw, K. Podsypanina, S. Bose, S. I. Wang, J. Puc, C. Miliaresis, L. Rodgers, R. McCombie, S. H. Bigner, B. C. Giovanella, M. Ittmann, B. Tycko, H. Hibshoosh, M. H. Wigler and R. Parsons (1997). "PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer." Science **275**(5308): 1943-1947.
Li L, O. S., Dahiya R. (2004). " DNA methylation in prostate cancer." Biochim Biophys Acta. **1704**(2): 87–102. .
Li, Q., X. Liu, R.-X. Hua, F. Wang, H. An, W. Zhang and J.-H. Zhu (2015) "Association of three 8q24 polymorphisms with prostate cancer susceptibility: evidence from a meta-analysis with 50,854 subjects." Scientific Reports **5**, 12069 DOI: 10.1038/srep12069.
li, Q., X. Liu, R.-X. Hua, F. Wang, H. An, W. Zhang and J.-H. Zhu (2015). "Association of three 8q24 polymorphisms with prostate cancer susceptibility: evidence from a meta-analysis with 50,854 subjects." Scientific Reports **5**: 12069.
Liang Cheng, R. M., David G Bostwick et al (2012). "Staging of prostate cancer." Histopathology **60**: 87-117.

Liang, Y. X., R. J. Mo, H. C. He, J. H. Chen, J. Zou, Z. D. Han, J. M. Lu, C. Cai, Y. R. Zeng, W. D. Zhong and C. L. Wu (2015). "Aberrant hypomethylation-mediated CD147 overexpression promotes aggressive tumor progression in human prostate cancer." Oncology Reports **33**(5): 2648-2654.
Lin, X., L. Qu, Z. Chen, C. Xu, D. Ye, Q. Shao, X. Wang, J. Qi, Z. Chen, F. Zhou, M. Wang, Z. Wang, D. He, D. Wu, X. Gao, J. Yuan, G. Wang, Y. Xu, G. Wang, P. Dong, Y. Jiao, J. Yang, J. Ou-Yang, H. Jiang, Y. Zhu, S. Ren, Z. Zhang, C. Yin, Q. Wu, Y. Zheng, A. R. Turner, S. Tao, R. Na, Q. Ding, D. Lu, R. Shi, J. Sun, F. Liu, S. L. Zheng, Z. Mo, Y. Sun and J. Xu (2012). "A novel Germline mutation in HOXB13 is associated with prostate cancer risk in Chinese men." The Prostate: n/a-n/a.
Lindberg, J., D. Klevebring, W. Liu, M. Neiman, J. Xu, P. Wiklund, F. Wiklund, I. G. Mills, L. Egevad and H. Grönberg (2013). "Exome Sequencing of Prostate Cancer Supports the Hypothesis of Independent Tumour Origins." European Urology **63**(2): 347-353.
Liu, W., B.-L. Chang, S. Cramer, P. P. Koty, T. Li, J. Sun, A. R. Turner, C. Von Kap-Herr, P. Bobby, J. Rao, S. L. Zheng, W. B. Isaacs and J. Xu (2007). "Deletion of a Small Consensus Region at 6q15, Including the MAP3K7 Gene, Is Significantly Associated with High-Grade Prostate Cancers." Clinical Cancer Research **13**(17): 5028-5033.
Liu, W., B. L. Chang, S. Cramer, P. P. Koty, T. Li, J. Sun, A. R. Turner, C. Kap-Herr, P. Bobby, J. Rao, S. L. Zheng, W. B. Isaacs and J. Xu (2007). "Deletion of a small consensus region at 6q15, including the MAP3K7 gene, is significantly associated with high-grade prostate cancers." Clinical Cancer Research **13**(17): 5028-5033.
Logtenberg, M. E. W. and J. Boonstra (2013). "Cancer stem cells and addicted cancer cells." Oncology Discovery **1**(1).
Luchman, H. A., H. Benediktsson, M. L. Villemaire, A. C. Peterson and F. R. Jirik (2008). "The pace of prostatic intraepithelial neoplasia development is determined by the timing of Pten tumor suppressor gene excision." PloS one **3**(12): e3940.
Lynch, H. T., O. Kosoko-Lasaki, S. W. Leslie, M. Rendell, T. Shaw, C. Snyder, A. V. D'Amico, S. Buxbaum, W. B. Isaacs, S. Loeb, J. W. Moul and I. Powell (2016). "Screening for familial and hereditary prostate cancer." International Journal of Cancer **138**(11): 2579-2591.
Maier, C., Z. Vesovic, N. Bachmann, K. Herkommer, A. K. Braun, H. M. Surowy, G. Assum, T. Paiss and W. Vogel (2006). "Germline mutations of the MSR1 gene in prostate cancer families from Germany." Human mutation **27**(1): 98-102.
Majewski, J., J. Schwartzentruber, E. Lalonde, A. Montpetit and N. Jabado (2011). "What can exome sequencing do for you?" Journal of Medical Genetics **48**(9): 580-589.
Malfitano, A. M., E. Ciaglia, G. Gangemi, P. Gazzerro, C. Laezza and M. Bifulco (2011). "Update on the endocannabinoid system as an anticancer target." Expert opinion on therapeutic targets **15**(3): 297-308.
Manson-Bahr, D., R. Ball, G. Gundem, K. Sethia, R. Mills, M. Rochester, V. Goody, E. Anderson, S. O'Meara, M. Flather, M. Keeling, M. Yazbek-Hanna, R. Hurst, H. Curley, J. Clark, D. S. Brewer, U. McDermott and C. Cooper (2015). "Mutation detection in formalin-fixed prostate cancer biopsies taken at the time of diagnosis using next-generation DNA sequencing." Journal of clinical pathology.
Mao, X., Y. Yu, L. K. Boyd, G. Ren, D. Lin, T. Chaplin, S. C. Kudahetti, E. Stankiewicz, L. Xue, L. Beltran, M. Gupta, R. T. Oliver, N. R. Lemoine, D. M. Berney, B. D. Young

and Y. J. Lu (2010). "Distinct genomic alterations in prostate cancers in Chinese and Western populations suggest alternative pathways of prostate carcinogenesis." Cancer research **70**(13): 5207-5212.
Mao, X., Y. Yu, L. K. Boyd, G. Ren, D. Lin, T. Chaplin, S. C. Kudahetti, E. Stankiewicz, L. Xue, L. Beltran, M. Gupta, R. T. D. Oliver, N. R. Lemoine, D. M. Berney, B. D. Young and Y. J. Lu (2010). "Distinct genomic alterations in prostate cancers in Chinese and Western populations suggest alternative pathways of prostate carcinogenesis." Cancer research **70**(13): 5207-5212.
Marzec, J., X. Mao, M. Li, M. Wang, N. Feng, X. Gou, G. Wang, Z. Sun, J. Xu, H. Xu, X. Zhang, S.-C. Zhao, G. Ren, Y. Yu, Y. Wu, J. Wu, Y. Xue, B. Zhou, Y. Zhang, X. Xu, J. Li, W. He, S. Benlloch, H. Ross-Adams, L. Chen, J. Li, Y. Hong, Z. Kote-Jarai, X. Cui, J. Hou, J. Guo, L. Xu, C. Yin, Y. Zhou, D. E. Neal, T. Oliver, G. Cao, Z. Zhang, D. F. Easton, C. Chelala, P. Consortium, C. Group, A. A. Al Olama, R. A. Eeles, H. Zhang and Y.-J. Lu (2016). "A genetic study and meta-analysis of the genetic predisposition of prostate cancer in a Chinese population." Oncotarget.
Marzec, J., X. Mao, M. Li, M. Wang, N. Feng, X. Gou, G. Wang, Z. Sun, J. Xu, H. Xu, X. Zhang, S.-C. Zhao, G. Ren, Y. Yu, Y. Wu, J. Wu, Y. Xue, B. Zhou, Y. Zhang, X. Xu, J. Li, W. He, S. Benlloch, H. Ross-Adams, L. Chen, J. Li, Y. Hong, Z. Kote-Jarai, X. Cui, J. Hou, J. Guo, L. Xu, C. Yin, Y. Zhou, D. E. Neal, T. Oliver, G. Cao, Z. Zhang, D. F. Easton, C. Chelala, T. P. Consortium, T. C. Group, A. A. A. Olama, R. A. Eeles, H. Zhang and Y.-J. Lu (2016). A genetic study and meta-analysis of the genetic predisposition of prostate cancer in a Chinese population.
Matsuda, L. A., S. J. Lolait, M. J. Brownstein, A. C. Young and T. I. Bonner (1990). "Structure of a cannabinoid receptor and functional expression of the cloned cDNA." Nature **346**(6284): 561-564.
McNeal, J. E. (1969). "Origin and development of carcinoma in the prostate." Cancer **23**(1): 24-34.
Mechoulam, R., S. Ben-Shabat, L. Hanus, M. Ligumsky, N. E. Kaminski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, D. R. Compton, R. G. Pertwee, G. Griffin, M. Bayewitch, J. Barg and Z. Vogel (1995). "Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors." Biochemical Pharmacology **50**(1): 83-90.
Mei Ka Fong, R. H., Anthony Jarkowski (2012). "A new era for castrate resistant prostate cancer: A treatment review and update." Journal of Oncology Pharmcy Practice: 1-12.
Melck, D., L. De Petrocellis, P. Orlando, T. Bisogno, C. Laezza, M. Bifulco and V. Di Marzo (2000). "Suppression of Nerve Growth Factor Trk Receptors and Prolactin Receptors by Endocannabinoids Leads to Inhibition of Human Breast and Prostate Cancer Cell Proliferation 1." Endocrinology **141**(1): 118-126.
Melck, D., L. D. Petrocellis, P. Orlando, T. Bisogno, C. Laezza, M. Bifulco and V. D. Marzo (2000). "Suppression of Nerve Growth Factor Trk Receptors and Prolactin Receptors by Endocannabinoids Leads to Inhibition of Human Breast and Prostate Cancer Cell Proliferation." Endocrinology **141**(1): 118-126.
Mellinger, G. T., D. Gleason and J. Bailar, 3rd (1967). "The histology and prognosis of prostatic cancer." The Journal of urology **97**(2): 331-337.
Meneu-Diaz, J. C., F. Bedoya, R. Enriquez-Salamanca, I. Vegh, A. M. Elola-Olaso, A. Marcacuzco, P. Rico-Selas and A. Moreno (2011). "Description of Mutation in CNR1 Gene and VEGF Expression in Esophageal Cancer." European journal of cancer **47**: S474-S474.

Messalli, E. M., F. Grauso, R. Luise, A. Angelini and R. Rossiello (2014). "Cannabinoid receptor type 1 immunoreactivity and disease severity in human epithelial ovarian tumors." American Journal of Obstetrics and Gynecology **211**(3): 234.e231-234.e236.
Micheal F Leitzmann, S. R. (2012). "Risk factors for the onset of prostatic cancer: age, ;ocation, and behavioral correlates." Clinical Epidermiology **4**: 1-11.
Miller, L. K. and L. A. Devi (2011). "The Highs and Lows of Cannabinoid Receptor Expression in Disease: Mechanisms and Their Therapeutic Implications." Pharmacological Reviews **63**(3): 461-470.
Mimeault, M., N. Pommery, N. Wattez, C. Bailly and J. P. Henichart (2003). "Anti-proliferative and apoptotic effects of anandamide in human prostatic cancer cell lines: Implication of epidermal growth factor receptor down-regulation and ceramide production." The Prostate **56**(1): 1-12.
Minelli, A., I. Bellezza, C. Conte and Z. Culig (2009). "Oxidative stress-related aging: A role for prostate cancer?" Biochimica et Biophysica Acta (BBA) - Reviews on Cancer **1795**(2): 83-91.
Mosquera, J.-M., S. Perner, E. M. Genega, M. Sanda, M. D. Hofer, K. D. Mertz, P. L. Paris, J. Simko, T. A. Bismar, G. Ayala, R. B. Shah, M. Loda and M. A. Rubin (2008). "Characterization of TMPRSS2-ERG fusion high-grade prostatic intraepithelial neoplasia and potential clinical implications." Clinical cancer research : an official journal of the American Association for Cancer Research **14**(11): 3380-3385.
Munro, S., K. L. Thomas and M. Abu-Shaar (1993). "Molecular characterization of a peripheral receptor for cannabinoids." Nature **365**(6441): 61-65.
Narla, G., K. E. Heath, H. L. Reeves, D. Li, L. E. Giono, A. C. Kimmelman, M. J. Glucksman, J. Narla, F. J. Eng, A. M. Chan, A. C. Ferrari, J. A. Martignetti and S. L. Friedman (2001). "KLF6, a candidate tumor suppressor gene mutated in prostate cancer." Science **294**(5551): 2563-2566.
Nelson, W. G., A. M. De Marzo and S. Yegnasubramanian (2009). "Epigenetic alterations in human prostate cancers." Endocrinology **150**(9): 3991-4002.
Nilsson, S. (2016). "Radionuclide Therapies in Prostate Cancer: Integrating Radium-223 in the Treatment of Patients With Metastatic Castration-Resistant Prostate Cancer." Current Oncology Reports **18**(2): 1-12.
Olea-Herrero, N., D. Vara, S. Malagarie-Cazenave and I. D√≠az-Laviada (2009). "Inhibition of human tumour prostate PC-3 cell growth by cannabinoids R()-Methanandamide and JWH-015: Involvement of CB 2." British journal of cancer **101**(6): 940-950.
Olea-Herrero, N., D. Vara, S. Malagarie-Cazenave and I. Díaz-Laviada (2009). "Inhibition of human tumour prostate PC-3 cell growth by cannabinoids R(+)-Methanandamide and JWH-015: Involvement of CB(2)." British Journal of Cancer **101**(6): 940-950.
Omlin, A., C. Pezaro and S. Gillessen Sommer (2014). "Sequential use of novel therapeutics in advanced prostate cancer following docetaxel chemotherapy." Therapeutic Advances in Urology **6**(1): 3-14.
Onaivi, E. S., C. M. Leonard, H. Ishiguro, P. W. Zhang, Z. Lin, B. E. Akinshola and G. R. Uhl (2002). "Endocannabinoids and cannabinoid receptor genetics." Progress in neurobiology **66**(5): 307-344.
ORELLANA-SERRADELL, O., C. POBLETE, C. SANCHEZ, E. CASTELL √ ìN, I. GALLEGOS, C. HUIDOBRO, M. LLANOS and H. CONTRERAS (2015). "Proapoptotic

effect of endocannabinoids in prostate cancer cells." Oncology Reports **33**(4): 1599.
Orellana-Serradell, O., C. E. Poblete, C. Sanchez, E. A. Castell√ìN, I. Gallegos, C. Huidobro, M. N. Llanos and H. R. Contreras (2015). "Proapoptotic effect of endocannabinoids in prostate cancer cells." Oncology Reports **33**(4): 1599-1608.
Pacher, P., S. BÅTkai and G. Kunos (2006). "The Endocannabinoid System as an Emerging Target of Pharmacotherapy." Pharmacological reviews **58**(3): 389-462.
Parker, B. C. and W. Zhang (2013). "Fusion genes in solid tumors: an emerging target for cancer diagnosis and treatment." Chin J Cancer **32**(11): 594-603.
Parker, C., S. Nilsson, D. Heinrich, S. I. Helle, J. O'Sullivan, S. D. Foss√ ·, A. Chodacki, P. Ç. Wiechno, J. Logue and M. Seke (2013). "Alpha emitter radium-223 and survival in metastatic prostate cancer." New England Journal of Medicine **369**(3): 213-223.
Pellerito, O., A. Notaro, S. Sabella, A. De Blasio, R. Vento, G. Calvaruso and M. Giuliano (2014). "WIN induces apoptotic cell death in human colon cancer cells through a block of autophagic flux dependent on PPARgamma down-regulation." Apoptosis : an international journal on programmed cell death **19**(6): 1029-1042.
Perletti, G., E. Monti, E. Marras, A. Cleves, V. Magri, A. Trinchieri and P. S. Rennie (2015). "Efficacy and safety of second-line agents for treatment of metastatic castration-resistant prostate cancer progressing after docetaxel. A systematic review and meta-analysis." Archivio Italiano di Urologia e Andrologia **87**(2): 121-129.
Pertwee, R. G. (2000). "Cannabinoid receptor ligands: clinical and neuropharmacological considerations, relevant to future drug discovery and development." Expert Opinion on Investigational Drugs **9**(7): 1553-1571.
Pertwee, R. G., A. C. Howlett, M. E. Abood, S. P. Alexander, V. Di Marzo, M. R. Elphick, P. J. Greasley, H. S. Hansen, G. Kunos, K. Mackie, R. Mechoulam and R. A. Ross (2010). "International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2)." Pharmacological reviews **62**(4): 588-631.
Petrovics G, L. A., Shaheduzzaman S, et al. (2005). "Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. ." Oncogene **24**(23): 3847-3852.
Petrylak , D. P., C. M. Tangen , M. H. A. Hussain , P. N. J. Lara , J. A. Jones , M. E. Taplin , P. A. Burch , D. Berry , C. Moinpour , M. Kohli , M. C. Benson , E. J. Small , D. Raghavan and E. D. Crawford (2004). "Docetaxel and Estramustine Compared with Mitoxantrone and Prednisone for Advanced Refractory Prostate Cancer." New England Journal of Medicine **351**(15): 1513-1520.
Pezaro, C. J., D. Mukherji and J. S. De Bono (2012). "Abiraterone acetate: redefining hormone treatment for advanced prostate cancer." Drug discovery today **17**(5): 221-226.
Pflueger, D., S. Terry, A. Sboner, L. Habegger, R. Esgueva, P.-C. Lin, M. A. Svensson, N. Kitabayashi, B. J. Moss, T. Y. MacDonald, X. Cao, T. Barrette, A. K. Tewari, M. S. Chee, A. M. Chinnaiyan, D. S. Rickman, F. Demichelis, M. B. Gerstein and M. A. Rubin (2011). "Discovery of non-ETS gene fusions in human prostate cancer using next-generation RNA sequencing." Genome Research **21**(1): 56-67.
Phay, J. E. and M. D. Ringel (2013). "Metastatic mechanisms in follicular cell-derived thyroid cancer." Endocrine-Related Cancer **20**(6): R307-R319.

Pisanti, S., P. Picardi, L. Prota, M. C. Proto, C. Laezza, P. G. McGuire, L. Morbidelli, P. Gazzerro, M. Ziche, A. Das and M. Bifulco (2011). Genetic and pharmacologic inactivation of cannabinoid CB1 receptor inhibits angiogenesis.
Porcella, A., G. Marchese, M. A. Casu, A. Rocchitta, M. L. Lai, G. L. Gessa and L. Pani (2002). "Evidence for functional CB1 cannabinoid receptor expressed in the rat thyroid." European journal of endocrinology / European Federation of Endocrine Societies **147**(2): 255-261.
Porter, A. C., J.-M. Sauer, M. D. Knierman, G. W. Becker, M. J. Berna, J. Bao, G. G. Nomikos, P. Carter, F. P. Bymaster, A. B. Leese and C. C. Felder (2002). "Characterization of a Novel Endocannabinoid, Virodhamine, with Antagonist Activity at the CB1 Receptor." Journal of Pharmacology and Experimental Therapeutics **301**(3): 1020-1024.
Preet, A., Z. Qamri, M. W. Nasser, A. Prasad, K. Shilo, X. Zou, J. E. Groopman and R. K. Ganju (2011). "Cannabinoid receptors, CB1 and CB2, as novel targets for inhibition of non-small cell lung cancer growth and metastasis." Cancer prevention research **4**(1): 65-75.
Preet, A., Z. Qamri, M. W. Nasser, A. Prasad, K. Shilo, X. Zou, J. E. Groopman and R. K. Ganju (2011). "Cannabinoid Receptors, CB1 and CB2, as Novel Targets for Inhibition of Non–Small Cell Lung Cancer Growth and Metastasis." Cancer Prevention Research **4**(1): 65-75.
Rabbani, B., M. Tekin and N. Mahdieh (2014). "The promise of whole-exome sequencing in medical genetics." J Hum Genet **59**(1): 5-15.
Ramer, R. and B. Hinz (2008). "Inhibition of cancer cell invasion by cannabinoids via increased expression of tissue inhibitor of matrix metalloproteinases-1." Journal of the National Cancer Institute **100**(1): 59-69.
Ramos, J. A. and F. J. Bianco (2012). "The role of cannabinoids in prostate cancer: Basic science perspective and potential clinical applications." Indian Journal of Urology : IJU : Journal of the Urological Society of India **28**(1): 9-14.
Ren, X.-Q., J.-G. Zhang, S.-Y. Xin, T. Cheng, L. Li and W.-H. Ren (2015). "Variants on 8q24 and prostate cancer risk in Chinese population: a meta-analysis." International Journal of Clinical and Experimental Medicine **8**(6): 8561-8570.
Rinaldi-Carmona, M., B. Calandra, D. Shire, M. Bouaboula, D. Oustric, F. Barth, P. Casellas, P. Ferrara and G. Le Fur (1996). "Characterization of two cloned human CB1 cannabinoid receptor isoforms." Journal of Pharmacology and Experimental Therapeutics **278**(2): 871-878.
Robinson, D., E. M. Van Allen, Y.-M. Wu, N. Schultz, R. J. Lonigro, J.-M. Mosquera, B. Montgomery, M.-E. Taplin, C. C. Pritchard and G. Attard (2015). "Integrative clinical genomics of advanced prostate cancer." Cell **161**(5): 1215-1228.
Ross, A., F. Feng, M. Ghadessi, N. Erho, A. Crisan, C. Buerki, D. Sundi, A. Mitra, I. Vergara and D. Thompson (2014). "A genomic classifier predicting metastatic disease progression in men with biochemical recurrence after prostatectomy." Prostate cancer and prostatic diseases **17**(1): 64-69.
Ruiz, L., A. Miguel and I. D√≠az-Laviada (1999). "Œî9-Tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism." FEBS letters **458**(3): 400-404.
Ruiz-Llorente, L., M. G. Sanchez, M. J. Carmena, J. C. Prieto, M. Sanchez-Chapado, A. Izquierdo and I. Diaz-Laviada (2003). "Expression of functionally active cannabinoid receptor CB1 in the human prostate gland." The Prostate **54**(2): 95-102.

Ryberg, E., H. K. Vu, N. Larsson, T. Groblewski, S. Hjorth, T. Elebring, S. Sj√ ∂ gren and P. J. Greasley (2005). "Identification and characterisation of a novel splice variant of the human CB1 receptor." FEBS letters **579**(1): 259-264.
Ryberg, E., H. K. Vu, N. Larsson, T. Groblewski, S. Hjorth, T. Elebring, S. Sjögren and P. J. Greasley (2005). "Identification and characterisation of a novel splice variant of the human CB1 receptor." FEBS Letters **579**(1): 259-264.
Salami, S. S., F. Schmidt, B. Laxman, M. M. Regan, D. S. Rickman, D. Scherr, G. Bueti, J. Siddiqui, S. A. Tomlins, J. T. Wei, A. Chinnaiyan, M. A. Rubin and M. G. Sanda (2013). "Combining Urinary Detection of TMPRSS2:ERG and PCA3 with Serum PSA to Predict Diagnosis of Prostate Cancer." Urologic oncology **31**(5): 566-571.
Salinas, C. A., E. Kwon, C. S. Carlson, J. S. Koopmeiners, Z. Feng, D. M. Karyadi, E. A. Ostrander and J. L. Stanford (2008). "Multiple Independent Genetic Variants in the 8q24 Region Are Associated with Prostate Cancer Risk." Cancer Epidemiology Biomarkers & Prevention **17**(5): 1203-1213.
Sanchez, M. G., L. Ruiz-Llorente, A. M. Sanchez and I. Diaz-Laviada (2003). "Activation of phosphoinositide 3-kinase/PKB pathway by CB(1) and CB(2) cannabinoid receptors expressed in prostate PC-3 cells. Involvement in Raf-1 stimulation and NGF induction." Cellular signalling **15**(9): 851-859.
Sarfaraz, S., V. M. Adhami, D. N. Syed, F. Afaq and H. Mukhtar (2008). "Cannabinoids for Cancer Treatment: Progress and Promise." Cancer Research **68**(2): 339-342.
Sarfaraz, S., F. Afaq, V. M. Adhami, A. Malik and H. Mukhtar (2006). "Cannabinoid receptor agonist-induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G1 cell cycle arrest." The Journal of biological chemistry **281**(51): 39480-39491.
Sarfaraz, S., F. Afaq, V. M. Adhami, A. Malik and H. Mukhtar (2006). "Cannabinoid Receptor Agonist-induced Apoptosis of Human Prostate Cancer Cells LNCaP Proceeds through Sustained Activation of ERK1/2 Leading to G1 Cell Cycle Arrest." Journal of Biological Chemistry **281**(51): 39480-39491.
Sarfaraz, S., F. Afaq, V. M. Adhami and H. Mukhtar (2005). "Cannabinoid receptor as a novel target for the treatment of prostate cancer." Cancer research **65**(5): 1635-1641.
Scher , H. I., K. Fizazi , F. Saad , M.-E. Taplin , C. N. Sternberg , K. Miller , R. de Wit , P. Mulders , K. N. Chi , N. D. Shore , A. J. Armstrong , T. W. Flaig , A. Fl√ ©chon , P. Mainwaring , M. Fleming , J. D. Hainsworth , M. Hirmand , B. Selby , L. Seely and J. S. de Bono (2012). "Increased Survival with Enzalutamide in Prostate Cancer after Chemotherapy." New England Journal of Medicine **367**(13): 1187-1197.
Scher, H. I., K. Fizazi, F. Saad, M.-E. Taplin, C. N. Sternberg, K. Miller, R. de Wit, P. Mulders, K. N. Chi, N. D. Shore, A. J. Armstrong, T. W. Flaig, A. Fléchon, P. Mainwaring, M. Fleming, J. D. Hainsworth, M. Hirmand, B. Selby, L. Seely and J. S. de Bono (2012). "Increased Survival with Enzalutamide in Prostate Cancer after Chemotherapy." New England Journal of Medicine **367**(13): 1187-1197.
Schumacher, F. R., S. I. Berndt, A. Siddiq, K. B. Jacobs, Z. Wang, S. Lindstrom, V. L. Stevens, C. Chen, A. M. Mondul, R. C. Travis, D. O. Stram, R. A. Eeles, D. F. Easton, G. Giles, J. L. Hopper, D. E. Neal, F. C. Hamdy, J. L. Donovan, K. Muir, A. A. Al Olama, Z. Kote-Jarai, M. Guy, G. Severi, H. Gr√ ∂ nberg, W. B. Isaacs, R. Karlsson, F. Wiklund, J. Xu, N. E. Allen, G. L. Andriole, A. Barricarte, H. Boeing, H. Bas Bueno-de-Mesquita, E. D. Crawford, W. R. Diver, C. A. Gonzalez, J. M. Gaziano, E. L. Giovannucci, M. Johansson, L. Le Marchand, J. Ma, S. Sieri, P. r. Stattin, M. J.

Stampfer, A. Tjonneland, P. Vineis, J. Virtamo, U. Vogel, S. J. Weinstein, M. Yeager, M. J. Thun, L. N. Kolonel, B. E. Henderson, D. Albanes, R. B. Hayes, H. Spencer Feigelson, E. Riboli, D. J. Hunter, S. J. Chanock, C. A. Haiman and P. Kraft (2011). "Genome-wide association study identifies new prostate cancer susceptibility loci." Human molecular genetics **20**(19): 3867-3875.
Shan, L. (2010). Investigation of the role of t(4;6) and 6q15 deletion in prostate cancer development and progression. phD, Queen Mary, University of London.
Shan, L., L. Ambroisine, J. Clark, R. J. Yanez-Munoz, G. Fisher, S. C. Kudahetti, J. Yang, S. Kia, X. Mao, A. Fletcher, P. Flohr, S. Edwards, G. Attard, J. De-Bono, B. D. Young, C. S. Foster, V. Reuter, H. Moller, T. D. Oliver, D. M. Berney, P. Scardino, J. Cuzick, C. S. Cooper and Y. J. Lu (2010). "The identification of chromosomal translocation, t(4;6)(q22;q15), in prostate cancer." Prostate cancer and prostatic diseases **13**(2): 117-125.
Shaneyfelt, T., R. Husein, G. Bubley and C. S. Mantzoros (2000). "Hormonal predictors of prostate cancer: A meta-analysis." Journal of Clinical Oncology **18**(4): 847-853.
Sharma, M., J. B. Hudson, H. Adomat, E. Guns and M. E. Cox (2014). "<i>In Vitro</i> Anticancer Activity of Plant-Derived Cannabidiol on Prostate Cancer Cell Lines." Pharmacology & Pharmacy **Vol.05No.08**: 15.
Shire, D., C. Carillon, M. Kaghad, B. Calandra, M. Rinaldi-Carmona, G. r. L. Fur, D. Caput and P. Ferrara (1995). "An Amino-terminal Variant of the Central Cannabinoid Receptor Resulting from Alternative Splicing." Journal of Biological Chemistry **270**(8): 3726-3731.
Silva, A. M. a. G. A. e. (2010). "Survival of patients with clinically localized prostate cancer." Rev Saude Publica **44**: 2-8.
Simon M Collin, R. M. M., Chris Metcalfe, David Gunnell, Peter C Albertsen, David Neal, Freddie Hamdy, Peter Stephens, J Athene Lane, and J. D. Rollo Moore (2008). "Prostate-cancer mortality in the USA and UK in 1975–2004:

an ecological study

." the Lancet **9**(May): 445-451.
Snchez, M. G., A. M. Snchez, L. Ruiz-Llorente and I. Daz-Laviada (2003). "Enhancement of androgen receptor expression induced by (R)-methanandamide in prostate LNCaP cells." FEBS letters **555**(3): 561-566.
Song, C. and A. C. Howlett (1995). "Rat brain cannabinoid receptors are N-linked glycosylated proteins." Life sciences **56**(23-24): 1983-1989.
Song, J., Z. Tao, X. Liu, S. Gong and L. Gan (2016). "Relationship between CYP17 gene polymorphisms and risk of prostate cancer." Genetics and molecular research: GMR **15**(1).
Spitz, F. and E. E. M. Furlong (2012). "Transcription factors: from enhancer binding to developmental control." Nat Rev Genet **13**(9): 613-626.
Spurgers, K. B., N. S. Chari, N. L. Bohnenstiehl and T. J. McDonnell (2006). "Molecular mediators of cell death in multistep carcinogenesis: a path to targeted therapy." Cell Death Differ **13**(8): 1360-1370.
Srivastava, B. K., R. Soni, A. Joharapurkar, K. V. V. M. Sairam, J. Z. Patel, A. Goswami, S. A. Shedage, S. S. Kar, R. P. Salunke, S. B. Gugale, A. Dhawas, P. Kadam, B. Mishra, N. Sadhwani, V. B. Unadkat, P. Mitra, M. R. Jain and P. R. Patel (2008). "Bioisosteric replacement of dihydropyrazole of 4S-(−)-3-(4-chlorophenyl)-N-methyl-N′ -[(4-chlorophenyl)-sulfonyl]-4-phenyl-4,5-dihydro-1H-pyrazole-1-caboxamidine

(SLV-319) a potent CB1 receptor antagonist by imidazole and oxazole." Bioorganic & Medicinal Chemistry Letters **18**(3): 963-968.
Srivastava, M., Y. Torosyan, M. Raffeld, O. Eidelman, H. B. Pollard and L. Bubendorf (2007). "ANXA7 expression represents hormone-relevant tumor suppression in different cancers." International Journal of Cancer **121**(12): 2628-2636.
Sternberg, C. N., D. P. Petrylak, O. Sartor, J. A. Witjes, T. Demkow, J.-M. Ferrero, J.-C. Eymard, S. Falcon, F. Calabrò and N. James (2009). "Multinational, double-blind, phase III study of prednisone and either satraplatin or placebo in patients with castrate-refractory prostate cancer progressing after prior chemotherapy: the SPARC trial." Journal of Clinical Oncology **27**(32): 5431-5438.
Storebjerg, T. M., S. Høyer, P. Kirkegaard, F. Bro, G. the LuCamp Study, T. F. Ørntoft, M. Borre and K. D. Sørensen (2016). "Prevalence of the HOXB13 G84E mutation in Danish men undergoing radical prostatectomy and its correlations with prostate cancer risk and aggressiveness." BJU international: n/a-n/a.
Stoyanova, T., A. R. Cooper, J. M. Drake, X. Liu, A. J. Armstrong, K. J. Pienta, H. Zhang, D. B. Kohn, J. Huang, O. N. Witte and A. S. Goldstein (2013). "Prostate cancer originating in basal cells progresses to adenocarcinoma propagated by luminal-like cells." Proceedings of the National Academy of Sciences of the United States of America **110**(50): 20111-20116.
Straiker, A., J. Wager-Miller, J. Hutchens and K. Mackie (2012). "Differential signalling in human cannabinoid CB1 receptors and their splice variants in autaptic hippocampal neurones." British Journal of Pharmacology **165**(8): 2660-2671.
Straiker, A., J. Wager-Miller and K. Mackie (2012). "The CB(1) cannabinoid receptor C-terminus regulates receptor desensitization in autaptic hippocampal neurones." British Journal of Pharmacology **165**(8): 2652-2659.
Sun, X., H. F. Frierson, C. Chen, C. Li, Q. Ran, K. B. Otto, B. L. Cantarel, R. L. Vessella, A. C. Gao, J. Petros, Y. Miura, J. W. Simons and J. T. Dong (2005). "Frequent somatic mutations of the transcription factor ATBF1 in human prostate cancer." Nature genetics **37**(4): 407-412.
Sutcliffe, S. and G. A. Colditz (2013). "Prostate cancer: is it time to expand the research focus to early-life exposures?" Nat Rev Cancer **13**(3): 208-518.
Sweeney, C. J., Y.-H. Chen, M. Carducci, G. Liu, D. F. Jarrard, M. Eisenberger, Y.-N. Wong, N. Hahn, M. Kohli, M. M. Cooney, R. Dreicer, N. J. Vogelzang, J. Picus, D. Shevrin, M. Hussain, J. A. Garcia and R. S. DiPaola (2015). "Chemohormonal Therapy in Metastatic Hormone-Sensitive Prostate Cancer." New England Journal of Medicine **373**(8): 737-746.
Takata, R., S. Akamatsu, M. Kubo, A. Takahashi, N. Hosono, T. Kawaguchi, T. Tsunoda, J. Inazawa, N. Kamatani, O. Ogawa, T. Fujioka, Y. Nakamura and H. Nakagawa (2010). "Genome-wide association study identifies five new susceptibility loci for prostate cancer in the Japanese population." Nature genetics **42**(9): 751-754.
Tan, M. H. E., J. Li, H. E. Xu, K. Melcher and E.-l. Yong (2015). "Androgen receptor: structure, role in prostate cancer and drug discovery." Acta Pharmacol Sin **36**(1): 3-23.
Tannock , I. F., R. de Wit , W. R. Berry , J. Horti , A. Pluzanska , K. N. Chi , S. Oudard , C. Th√ ©odore , N. D. James , I. Turesson , M. A. Rosenthal and M. A. Eisenberger (2004). "Docetaxel plus Prednisone or Mitoxantrone plus Prednisone for

Advanced Prostate Cancer." New England Journal of Medicine **351**(15): 1502-1512.
Taylor, B. S., N. Schultz, H. Hieronymus, A. Gopalan, Y. Xiao, B. S. Carver, V. K. Arora, P. Kaushik, E. Cerami, B. Reva, Y. Antipin, N. Mitsiades, T. Landers, I. Dolgalev, J. E. Major, M. Wilson, N. D. Socci, A. E. Lash, A. Heguy, J. A. Eastham, H. I. Scher, V. E. Reuter, P. T. Scardino, C. Sander, C. L. Sawyers, W. L. Gerald and M. P. C. O. G. for the (2010). "Integrative genomic profiling of human prostate cancer." Cancer cell **18**(1): 11-22.
Taylor, R. A., R. Toivanen, M. Frydenberg, J. Pedersen, L. Harewood, A. T. Collins, N. J. Maitland, G. P. Risbridger and A. P. C. Bioresource (2012). "Human epithelial basal cells are cells of origin of prostate cancer, independent of CD133 status." Stem Cells **30**(6): 1087-1096.
Teoh, J. Y. C., S. K. K. Yuen, J. H. L. Tsu, C. K. W. Wong, B. S. H. Ho, A. T. L. Ng, W.-K. Ma, K.-L. Ho and M.-K. Yiu (2015). "Prostate cancer detection upon transrectal ultrasound-guided biopsy in relation to digital rectal examination and prostate-specific antigen level: what to expect in the Chinese population?" Asian journal of andrology **17**(5): 821-825.
Theodorescu, D. (2010). " Prostate Cancer - Biology, Diagnosis, Pathology, Staging, and Natural History."
Thomas, G., K. B. Jacobs, M. Yeager, P. Kraft, S. Wacholder, N. Orr, K. Yu, N. Chatterjee, R. Welch, A. Hutchinson, A. Crenshaw, G. Cancel-Tassin, B. J. Staats, Z. Wang, J. Gonzalez-Bosquet, J. Fang, X. Deng, S. I. Berndt, E. E. Calle, H. S. Feigelson, M. J. Thun, C. Rodriguez, D. Albanes, J. Virtamo, S. Weinstein, F. R. Schumacher, E. Giovannucci, W. C. Willett, O. Cussenot, A. Valeri, G. L. Andriole, E. D. Crawford, M. Tucker, D. S. Gerhard, J. F. Fraumeni, R. Hoover, R. B. Hayes, D. J. Hunter and S. J. Chanock (2008). "Multiple loci identified in a genome-wide association study of prostate cancer." Nature genetics **40**(3): 310-315.
Tombal, B. and F. Lecouvet (2012). "Modern Detection of Prostate Cancer's Bone Metastasis: Is the Bone Scan Era Over?" Advances in Urology **2012**: 8.
Tomlins, S. A. (2014). "Urine PCA3 and TMPRSS2:ERG Using Cancer-specific Markers to Detect Cancer." European urology **65**(3): 543-545.
Tomlins SA, R. D., Perner S, et al. (2005). "Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. ." Science **310**(5748): 644-648.
Turati, F., E. Negri and C. L. Vecchia (2014). "Family history and the risk of cancer: genetic factors influencing multiple cancer sites." Expert review of anticancer therapy **14**(1): 1-4.
Turu, G. and L. Hunyady (2010). "Signal transduction of the CB1 cannabinoid receptor." Journal of Molecular Endocrinology **44**(2): 75-85.
UK, C. R.
Van den Broeck, T., S. Joniau, L. Clinckemalie, C. Helsen, S. Prekovic, L. Spans, L. Tosco, H. Van Poppel and F. Claessens (2014). "The Role of Single Nucleotide Polymorphisms in Predicting Prostate Cancer Risk and Therapeutic Decision Making." BioMed Research International **2014**: 16.
Van Dross, R., E. Soliman, S. Jha, T. Johnson and S. Mukhopadhyay (2013). "Receptor-dependent and receptor-independent endocannabinoid signaling: A therapeutic target for regulation of cancer growth." Life sciences **92**(8,Äì9): 463-466.

van Leenders, G. J. L. H., T. W. Aalders, C. A. Hulsbergen-van de Kaa, D. J. Ruiter and J. A. Schalken (2001). "Expression of basal cell keratins in human prostate cancer metastases and cell lines." The Journal of Pathology **195**(5): 563-570.
Vasiljević, N., K. Wu, A. R. Brentnall, D. C. Kim, M. A. Thorat, S. C. Kudahetti, X. Mao, L. Xue, Y. Yu, G. L. Shaw, L. Beltran, Y.-J. Lu, D. M. Berney, J. Cuzick and A. T. Lorincz (2011). "Absolute quantitation of DNA methylation of 28 candidate genes in prostate cancer using pyrosequencing." Disease Markers **30**(4): 151-161.
Velasco, G., S. Hernández-Tiedra, D. Dávila and M. Lorente (2015). "The use of cannabinoids as anticancer agents." Progress in Neuro-Psychopharmacology and Biological Psychiatry(0).
Velasco, G., C. Sanchez and M. Guzm √ ° n (2012). "Towards the use of cannabinoids as antitumour agents." Nat Rev Cancer **12**(6): 436-444.
Verhagen, P. C. M. S., K. G. L. Hermans, M. O. Brok, W. M. van Weerden, M. G. J. Tilanus, R. A. de Weger, T. A. Boon and J. Trapman (2002). "Deletion of chromosomal region 6q14-16 in prostate cancer." International Journal of Cancer **102**(2): 142-147.
Vinall, R. L., J. Q. Chen, N. E. Hubbard, S. S. Sulaimon, M. M. Shen, R. W. DeVere White and A. D. Borowsky (2012). "Initiation of prostate cancer in mice by Tp53R270H: evidence for an alternative molecular progression." Disease Models & Mechanisms **5**(6): 914-920.
Vogelstein, B., N. Papadopoulos, V. E. Velculescu, S. Zhou, L. A. Diaz and K. W. Kinzler (2013). "Cancer Genome Landscapes." Science **339**(6127): 1546-1558.
Wang, D., H. Wang, W. Ning, M. G. Backlund, S. K. Dey and R. N. DuBois (2008). "Loss of Cannabinoid Receptor 1 Accelerates Intestinal Tumor Growth." Cancer research **68**(15): 6468-6476.
Wang, Z.-Y., H.-Y. Li, Z. Jiang, T.-B. Zhou and G. P. C. Drummen (2015). "GSTM1 Gene Polymorphism is Implicated in Increased Susceptibility to Prostate Cancer in Caucasians and Asians." Technology in Cancer Research & Treatment.
Ware, M. A., C. R. Doyle, R. Woods, M. E. Lynch and A. J. Clark (2003). "Cannabis use for chronic non-cancer pain: results of a prospective survey." Pain **102**(1,Äì2): 211-216.
Wei, B., Z. Xu, J. Ruan, M. Zhu, K. Jin, D. Zhou, Z. Yan, F. Xuan, H. Zhou, X. Huang, J. Zhang, P. Lu and J. Shao (2012). "RNASEL Asp541Glu and Arg462Gln polymorphisms in prostate cancer risk: evidences from a meta-analysis." Molecular biology reports **39**(3): 2347-2353.
Weigelt, B., J. L. Peterse and L. J. van't Veer (2005). "Breast cancer metastasis: markers and models." Nat Rev Cancer **5**(8): 591-602.
William K. Oh, J. L. (2007.). Prostate Cancer, William K Oh, J.L.
Williams, J. L., P. A. Greer and J. A. Squire (2014). "Recurrent copy number alterations in prostate cancer: an in silico meta-analysis of publicly available genomic data." Cancer genetics **207**(10,Äì12): 474-488.
Williams, K. A., M. Lee, Y. Hu, J. Andreas, S. J. Patel, S. Zhang, P. Chines, A. Elkahloun, S. Chandrasekharappa, J. S. Gutkind, A. A. Molinolo and N. P. S. Crawford (2014) "A systems genetics approach identifies CXCL14, ITGAX, and LPCAT2 as novel aggressive prostate cancer susceptibility genes." PLoS genetics **10**, e1004809 DOI: 10.1371/journal.pgen.1004809.
Wilt, T. J. and I. M. Thompson (2006). "Clinically localised prostate cancer." BMJ **333**(7578): 1102-1106.

Witte, J. S. (2007). "Multiple prostate cancer risk variants on 8q24." Nature genetics **39**(5): 579-580.
Wu, G., S. Huang, K. L. Nastiuk, J. Li, J. Gu, M. Wu, Q. Zhang, H. Lin and D. Wu (2015). "Variant allele of HSD3B1 increases progression to castration‚Äêresistant prostate cancer." The Prostate **75**(7): 777-782.
Wu, M., L. Shi, A. Cimic, L. Romero, G. Sui, C. J. Lees, J. M. Cline, D. F. Seals, J. S. Sirintrapun, T. P. McCoy, W. Liu, J. W. Kim, G. A. Hawkins, D. M. Peehl, J. Xu and S. D. Cramer (2012). "Suppression of tak1 promotes prostate tumorigenesis." Cancer research **72**(11): 2833-2843.
Xian, X.-S., H. Park, Y. K. Cho, I. S. Lee, S. W. Kim, M.-G. Choi, I.-S. Chung, K.-H. Han and J. M. Park (2010). "Effect of a synthetic cannabinoid agonist on the proliferation and invasion of gastric cancer cells." Journal of Cellular Biochemistry **110**(2): 321-332.
Xu, B., N. Tong, J. m. Li, Z. d. Zhang and H. f. Wu (2010). "ELAC2 polymorphisms and prostate cancer risk: a meta-analysis based on 18 case‚Äìcontrol studies." Prostate cancer and prostatic diseases **13**(3): 270-277.
Xu, J., Z. Mo, D. Ye, M. Wang, F. Liu, G. Jin, C. Xu, X. Wang, Q. Shao, Z. Chen, Z. Tao, J. Qi, F. Zhou, Z. Wang, Y. Fu, D. He, Q. Wei, J. Guo, D. Wu, X. Gao, J. Yuan, G. Wang, Y. Xu, G. Wang, H. Yao, P. Dong, Y. Jiao, M. Shen, J. Yang, J. Ou-Yang, H. Jiang, Y. Zhu, S. Ren, Z. Zhang, C. Yin, X. Gao, B. Dai, Z. Hu, Y. Yang, Q. Wu, H. Chen, P. Peng, Y. Zheng, X. Zheng, Y. Xiang, J. Long, J. Gong, R. Na, X. Lin, H. Yu, S. Tao, J. Feng, J. Sun, W. Liu, A. Hsing, J. Rao, Q. Ding, F. Wiklund, H. Gronberg, X.-O. Shu, W. Zheng, H. Shen, L. Jin, R. Shi, D. Lu, X. Zhang, J. Sun, S. L. Zheng and Y. Sun (2012). "Genome-wide association study in Chinese men identifies two new prostate cancer risk loci at 9q31.2 and 19q13.4." Nature genetics **44**(11): 1231-1235.
Xu, X. D., Y. Liu, S. F. Huang, G. X. Liu, C. Z. Xie, J. Zhou, W. T. Fan, Q. L. Li, Q. W. Wang, D. W. Zhong and X. Y. Miao (2006). "Overexpression of cannabinoid receptors CB1 and CB2 correlates with improved prognosis of patients with hepatocellular carcinoma." Cancer genetics and cytogenetics **171**(1): 31-38.
Xue, L., X. Mao, G. Ren, E. Stankiewicz, S. C. Kudahetti, D. Lin, L. Beltran, D. M. Berney and Y.-J. Lu (2012). "Chinese and Western prostate cancers show alternate pathogenetic pathways in association with ERG status." American journal of cancer research **2**(6): 736-744.
Yadav, S. S., J. Li, H. J. Lavery, K. K. Yadav and A. K. Tewari (2015). "Next-generation sequencing technology in prostate cancer diagnosis, prognosis, and personalized treatment." Urologic Oncology: Seminars and Original Investigations **33**(6): 267.e261-267.e213.
Yamada, T., M. Nakayama, T. Shimizu, S. Nonen, Y. Nakai, K. Nishimura, Y. Fujio, A. Okuyama, J. Azuma and N. Nonomura (2012). "Genetic polymorphisms of CYP17A1 in steroidogenesis pathway are associated with risk of progression to castration-resistant prostate cancer in Japanese men receiving androgen deprivation therapy." International Journal of Clinical Oncology **18**(4): 711-717.
Yang, Z., L. Yu and Z. Wang (2016). "PCA3 and TMPRSS2-ERG gene fusions as diagnostic biomarkers for prostate cancer." Chinese Journal of Cancer Research **28**(1): 65-71.
Yeager, M., N. Chatterjee, J. Ciampa, K. B. Jacobs, J. Gonzalez-Bosquet, R. B. Hayes, P. Kraft, S. Wacholder, N. Orr, S. Berndt, K. Yu, A. Hutchinson, Z. M. Wang, L. Amundadottir, H. S. Feigelson, M. J. Thun, W. R. Diver, D. Albanes, J. Virtamo, S. Weinstein, F. R. Schumacher, G. Cancel-Tassin, O. Cussenot, A. Valeri, G. L.

Andriole, E. D. Crawford, C. A. Haiman, B. Henderson, L. Kolonel, L. Le Marchand, A. Siddiq, E. Riboli, T. J. Key, R. Kaaks, W. Isaacs, S. Isaacs, K. E. Wiley, H. Gronberg, F. Wiklund, P. Stattin, J. F. Xu, S. L. Zheng, J. L. Sun, L. J. Vatten, K. Hveem, M. Kumle, M. Tucker, D. S. Gerhard, R. N. Hoover, J. F. Fraumeni, D. J. Hunter, G. Thomas and S. J. Chanock (2009). "Identification of a new prostate cancer susceptibility locus on chromosome 8q24." Nature genetics **41**(10): 1055-1057.
Yeager, M., N. Orr, R. B. Hayes, K. B. Jacobs, P. Kraft, S. Wacholder, M. J. Minichiello, P. Fearnhead, K. Yu, N. Chatterjee, Z. Wang, R. Welch, B. J. Staats, E. E. Calle, H. S. Feigelson, M. J. Thun, C. Rodriguez, D. Albanes, J. Virtamo, S. Weinstein, F. R. Schumacher, E. Giovannucci, W. C. Willett, G. Cancel-Tassin, O. Cussenot, A. Valeri, G. L. Andriole, E. P. Gelmann, M. Tucker, D. S. Gerhard, J. F. Fraumeni, R. Hoover, D. J. Hunter, S. J. Chanock and G. Thomas (2007). "Genome-wide association study of prostate cancer identifies a second risk locus at 8q24." Nature genetics **39**(5): 645-649.
Yoshimoto, M., J. C. Cutz, P. A. Nuin, A. M. Joshua, J. Bayani, A. J. Evans, M. Zielenska and J. A. Squire (2006). "Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias." Cancer genetics and cytogenetics **169**(2): 128-137.
Yoshimoto, M., O. Ludkovski, D. DeGrace, J. L. Williams, A. Evans, K. Sircar, T. A. Bismar, P. Nuin and J. A. Squire (2012). "PTEN genomic deletions that characterize aggressive prostate cancer originate close to segmental duplications." Genes, chromosomes & cancer **51**(2): 149-160.
Zhang, P. W., H. Ishiguro, T. Ohtsuki, J. Hess, F. Carillo, D. Walther, E. S. Onaivi, T. Arinami and G. R. Uhl (2004). "Human cannabinoid receptor 1: 5[prime] exons, candidate regulatory regions, polymorphisms, haplotypes and association with polysubstance abuse." Mol Psychiatry **9**(10): 916-931.
Zhu, H. S., J. F. Zhang, J. D. Zhou, M. J. Zhang and H. X. Hu (2015) "Association between the 8q24 rs6983267 T/G polymorphism and prostate cancer risk: a meta-analysis." Genetics and molecular research : GMR **14**, 19329-19341 DOI: 10.4238/2015.december.29.43.
Zogopoulos, P. (2015). "Cancer therapy-The role of cannabinoids and endocannabinoids." Cancer Cell & Microenvironment **2**(1).